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Isolation And Characterization Of Natural Antimicrobials From Plant And Marine Organisms

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DEDICATION

This manuscript is dedicated to my love, Jesse Goldstein.

Your patience and support were vital in the completion of this work.

Also, to my amazing parents, Aidan Canning and Valerie Clark

for teaching me early on that I could accomplish anything I set my mind to.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Introduction

The development of antimicrobial resistance by bacterial pathogens is a global crisis of increasing importance [1]. Scientists now recognize that each new antibiotic produced has a limited lifespan and therefore new anti-infective agents are constantly in demand. Throughout history, cultures have relied heavily on plants for their therapeutic properties. Many of the plants used in traditional medicine are still in use to this day [2]. Reports have estimated that plants have contributed to the development of at least half of current pharmaceuticals [3]. However, few of these are used as antimicrobials, and the vast majority are from bacterial and fungal sources [2]. This has prompted the scientific community to search for phytochemicals which can be used to develop pharmacetucal treatments for disease and illness-causing bacterial pathogens. With an estimated 250,000 to 500,000 plant species on earth, plant based antimicrobials represent a vast untapped reservoir with great pharmaceutical potential [2]. Furthermore, marine organisms account for about half of the worlds biodiversity and have been identified as an invaluable source of bioactive compounds as well. Although numerous marine organisms and sponges have been found to exhibit antimicrobial behaviour, antimicrobial activity of many marine organisms has yet to be explored [4]. The isolation and characterization of natural antimicrobials from plant and marine sources may lead to the development of antimicrobial agents to help control antimicrobial-resistant pathogens.

Antimicrobial Resistance

Antibiotic therapy is often the initial action taken against microbial infections in humans although a positive correlation is known to exist between antibiotic use and antimicrobial

resistance development [5]. Furthermore, the incidence of antimicrobial resistance in food-poisoning bacteria has also increased, partly due to the excessive use of antibiotic therapy in healthy livestock [6]. There is significant public demand for reformed prescribing practices by physicians and the cessation of unnecessary feeding of low levels of antibiotic to healthy food-producing animals [6]. Although informed physcians have improved prescribing practices, economic gain represents a strong motivator to farmers to continue to over-use antibiotics. Resistance in common pathogens has now made some of the most effective and reliable antimicrobial agents obsolete [1]. Of particular concern are those organisms which have developed resistance to several classes of antibiotics. Infections which were once easily cured may now require several courses of antibiotics and treatment may take months or years. In the past, pharmacetucial coprorations were consistently producing new, more potent antimicrobial agents in order to compete with the development of antimicrobial resistance. Although the need for new antimicrobials is apparent, pharmaceutical corporations are no longer developing sufficient amounts of novel antimicrobials and bacterial strains have developed resistance to even the newest antibiotics [7]. It is clear that without safe, effective alternatives readily available, antimicrobial resistance has the potential to hinder our ability to treat serious infections [5].

Relevant Bacterial Species

There are a number of bacterial species that are currently regarded as high priority pathogens based on antimicrobial resistance development, high incidence of infection, or a combination of both. Several bacterial species account for the majority of the cases of food borne illness and outbreaks. *Bacillus subtilis* is widespread in the environment, and can cause food spoilage and food borne illness even after cooking due to its ability to form spores [8].

Campylobacter jejuni is the leading cause of food borne bacterial gastroenteritis worldwide [9]. *Clostridium perfringens* food poisoning ranks among the most common foodborne illnesses worldwide [10]. *Escherichia coli* 0157 H:7 is responsible for many food and water borne outbreaks of diarrhea and hemorrhagic colitis worldwide [11]. *Listeria Monocytogenes* is a ubiquitous pathogen that is responsible for food borne outbreaks, often affecting immunocompromised individuals, pregnant women and newborns [12]. *Salmonella enterica* serovar typhimurium is a common cause of bacterial enterocolitis. Furthermore, testing has shown that an increasing proportion of isolates of *Salmonella enterica* are now resistant to several antimicrobial agents [13].

There are also a number of bacterial pathogens which contribute to many of the cases of bacterial infections in humans. Strains of the pathogenic bacteria *Acinetobacter baumanii* have been reported to be resistant to all known antibiotics [14]. *Clostridium difficile* is a bacterial species often associated with hospital outbreaks, due to the prevalent use of broad-spectrum antibiotics. One fluoroquinolone-resistant strain has been identified as the cause of numerous outbreaks of severe *C. difficile* infection [15]. Carbapenem reisitant strains of *Klebsiella pneumoniae;* a major cause of nosocomial infections, have left few treatment options for the infection [16]. Treatment of infections caused by the pathogenic bacteria *Pseudomonas Aeruginosa* is becoming more difficult due to its rapidly developing resistance to multiple classes of antibiotics [17]. Methycillin-resistant *Staphylococcus areus* (MRSA) infections are a major cause of hospital-acquired infections, and is now considered an epidemic in many US hospitals and long term care facilities [18]. *Streptococcus pneumoniae* is a human pathogen currently exhibiting fluoroquinolone resistance and representing a major cause of morbidity and mortality worldwide via diseases such as pneumonia, meningitis, and bacteraemia [19]. These

twelve bacterial species pose a significant risk to the public and intervention is currently required to regain control over these common pathogens.

Natural Products in Drug Discovery

With the increase in bacterial resistance to conventional treatments, there is also an increasing interest in plant-derived antimicrobial compounds. The screening of natural sources has been key in the discovery of many classes of drugs. Natural products, including both extracts as well as pure compounds have unlimited potential as new drug leads due to their tremendous chemical diversity [20]. Plant and marine sources have been found to be a limitless source of bioactive compounds [4]. The distribution of plant compounds is commonplace in natural food stores, and consumer demand for alternative treatments is increasing [2]. There was a reported 37% increase in the sale of plant derived medicines between the years 1995 to 1996 [21]. Unfortunately, the purity of these substances is unreliable and there is limited information available regarding dosage. Many scientists believe that natural antimicrobials may eventually replace traditional approaches of controlling pathogens [22]. The reasons to further develop natural products are numerous and include the high cost of currently available synthetic medicines, adverse side-effects of pharmaceuticals, and the public perception of gentleness of natural medicines.

The traditional method of compound isolation and characterization has long been used by pharmaceutical companies for natural product development. However, the process eventually acquired as reputation for being expensive, repetitive, tedious, and time consuming, causing pharmaceutical companies to draw back on these research approaches in the 1990's and early 2000's. At this time a new method was developed which relied on combinatorial chemistry and large compound libraries. The success of this method has been limited, which is proven by the

fact that only one combinatorial new entity has been approved by the FDA since the 1980's [23]. The public has relied on plants for new lead compounds in the initial stages of pharmaceutical development, and the combinatorial chemistry approach has not yet shown to be productive. It is unclear as to whether or not pharmaceutical companies and researchers will revert to the traditional method of isolation and characterization to increase the amount of new pharmaceuticals introduced to the market. It is important to note, however, that there have been numerous technological advances making the traditional method a more viable option. These include more sensitive instruments and more available NMR/MS data.

Natural Products as Antimicrobials

Natural products have been significant in antibiotic drug discovery with most antibiotics being derived from a natural product or a natural product lead [24]. Reports estimate that 60% of commercially available anti-infective agents are of natural product origin [3, 25]. Plants produce both primary and secondary metabolites. Those metabolites responsible for their defense mechanism fall under the latter category. Secondary metabolites are produced by plants for the purpose of protection against predators as well as infections [26]. Secondary metabolites such as phenolics, terpenoids, alkaloids, and flavonoids have known antimicrobial properties [2]. Similarly, marine sponges have developed chemical defenses to protect against the colonization of pathogenic bacteria [27]. For this reason, the antimicrobial activity of marine sponges has been studied in an attempt to harness the sponges natural potency [28-30]. Marine sources have been found to be an invaluable source of bioactive compounds [4].

Natural Products in Food Safety

Characterization of bioactive properties of plant extracts has potential application, not only in pharmaceuticals, but in agriculture and food preservation as well [31-33]. Currently, the most common application of natural antimicrobials is the direct addition of antimicrobial compounds to food [34]. Microorganisms can cause off flavor, odor, color, sensory, and textural properties in foods. Furthermore, microorganisms in food can cause food-borne illness [35]. Consumer concern regarding synthetic chemical additives has made foods preserved with natural additives the more popular choice [34]. For this reason, scientists have expanded their efforts to discover natural compounds that can prevent or inhibit the growth of microorganisms in food. Some natural antimicrobials are now used industrially in the processing of food. Foods are often dipped, sprayed, or coated with active solutions prior to packaging. This type of application of natural antimicrobial compounds is now utilized on a variety of foods including meat, fish, dairy products, minimally processed fruit and vegetables, and cereal-based products [34].

In this study, the antimicrobial activity of a number of plant and marine extracts is investigated. A number of extracts are chosen and the active compound(s) isolated and identified. The compounds are thoroughly investigated using a variety of techniques intended to evaluate each compound’s antimicrobial activity level, stability, safety, and overall potential for use as a natural antimicrobial.

CHAPTER 2: ISOLATION AND IDENTIFICATION OF COMPOUNDS FROM PLANTAND MARINE ORGANISMS WITH ANTIMICROBIAL ACTIVITY

Screening of Plant and Marine Organisms

Introduction

The natural products repository of the National Cancer Institute (NCI) through the U.S. National Institutes of Health (NIH) has collected close to 17,000 extracts of plant and marine organisms. Extracts were provided by the National Cancer Institute (NCI) to allow for this research to take place. Initial screening of antibacterial compounds from plants is commonly performed on crude extracts in order to identify 'hits', which are selected based on their desired bioactivity. In order to identify antimicrobial agents, the most commonly used screening method is known as broth microdilution [36]. This method allows for the testing of multiple samples at once. Although the this method is used numerous times throughout this work, at the initial screening stage, the method was simplified in order to test hundreds of plant and marine extracts at a single concentration, which could be repeated to test a number of different bacterial species.

Materials and Methods

Bacterial species- Seven high priority pathogens were used to fulfill this aim. Bacterial reference strains were obtained from the American Type Culture Collections (ATCC, Mannassas, VA). The bacterial strains included were: *A. baumannii*, ATCC 19606; *C. jejuni*, ATCC 33291; *C. difficile*, ATCC 9689; *C. Perfringens*, ATCC 13124; *K. pneumoniae*, ATCC 13883; *S. pneumoniae*, ATCC 49136; and *P. Aeruginosa*, ATCC 27853.

Plant and marine extracts- A total of 106 extracts of plant and marine organisms obtained from the natural products repository of NCI were identified through preliminary experiments on thousands of extracts which were chosen based on their inhibitory activity on *E.*

coli 0157 H:7 bacteria. Each extract was prepared by the Natural Products Extraction Laboratory by extracting each organism with a 1:1 mixture of dichloromethane and methanol and then with water. Each extract was stored at -20 ˚C.

Antimicrobial screening- A simplified version of the broth microdilution method described by the Clincal and Laboratory Standards Institute (CLSI) guidelines was utilized for the purpose of screening the extracts [37, 38]. Brain heart infusion broth (BHI), thioglycollate broth, nutrient broth, and cation-adjusted mueller hinton broth (CAMHB) were used for the growth medium. Each plant extract, was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution with the concentration of 1 mg/ml. This was further diluted in sterile distilled water to prepare test solutions with a concentration of 50 µg/ml. Overnight bacterial broth suspension was diluted 20x in an appropriate broth. Assay plates were prepared to contain extracts at a single concentration; *A. baumannii*; 10 µg/ml, *C. jejuni*; 5 µg/ml, *C. difficile* 5 µg/ml; *C. Perfringens;* 5 µg/ml, *K. pneumoniae*; 5 µg/ml, *S. pneumoniae*; 5 µg/ml, and *P. Aeruginosa;* 20 µg/ml. Assay plates were incubated in appropriate conditions (aerobic, anaerobic, or microaerophilic) at 37 ºC for 24 h, and growth or no-growth was assessed by the naked eye through comparison with controls. DMSO dilutions in sterile distilled water (1:19 ratio DMSO to water) that did not contain any test material and a blank containing no bacteria were included as controls.

Results

Screening of 106 plant and marine organism extracts led to the identification of 4 extracts with superior antimicrobial activity. The majority of the extracts tested had limited antimicrobial activity; inhibiting the growth of only one or none of the tested microorganisms. The extracts showing the greatest antimicrobial activity inhibited the growth of a minimum of 3

bacterial species. These extracts were N014267 from *Mammea africana,* C025163 from *Phyllospongia papyracea,* C022687 from *Monanchora unguiculata,* and C022615 from *Dysidea herbacea* (Tables 1a-c). These 4 extracts demonstrated activity against a broad spectrum of bacterial species. The *M. africana* extract inhibited the growth of *C. difficile, C. jejuni,* and *S. Pneumonoiae* at a concentration of 5 μg/ml. The *P. papyracea* extract also inhibited the growth of *C. difficile*, *C. jejuni*, and *S. Pneumonoiae* at a concentration of 5 μg/ml. *Monanchora unguiculata* is especially noteworthy as this extract showed inhibitory activity on every bacterial species included in the screening. Furthermore the *M. unguiculata* extract was the only extract of the 106 initial extracts to exhibit inhibitory activity on the bacterial species *A. baumani* and *P. aeruginosa*. Finally, the *D. herbacea* extract inhibited the growth of *C. difficile, C. jejuni, C. perfringens,* and *S. pneumoniae* at a concentration of 5 μg/ml. Based on the results of the initial screening, these 4 extracts were chosen for further study as they showed the strongest, broad-spectrum antimicrobial activity.

Table 1a *Antimicrobial activity of extracts numbered 1-40 on bacterial species.*

Extract ID Number		Bacterial Strains						
		A.baumani 10 µg/ml	C. difficile 5 µg/ml	C. jejuni 5 µg/ml	C. Perfringens 5 ug/ml	K. Pneumoniae 5 µg/ml	P. Aeruginosa 20 µg.ml	S. Pneumoniae 5 µg/ml
1	N043835	-	-	-	-	-	-	-
2	C021427	-	-	-	-	-	-	-
3	N104805	-	-	-	-	-	-	-
4	N107391	-	-	-	-	-	-	-
5	N010113	-	-	-	-	-	-	-
6	N014035	-	-	-	-	-	-	-
7	N009751	-	-	-	-	-	-	-
8	N105255	-	-	-	-	-	-	-
9	N009749	-	-	-	-	-	-	-
10	N009729	-	-	-	-	-	-	-
11	N009743	-	-	-	+	-	-	+
12	C025277	-	-	-	-	-	-	-
13	C022513	-	-	-	-	-	-	-
14	J003737	-	-	-	-	-	-	-
15	C023995	-	-	-	-	-	-	-
16	C025119	-	-	-	-	-	-	-
17	C021729	-	-	-	-	-	-	-
18	C022713	-	-	-	-	-	-	-
19	J003635	-	-	-	-	-	-	-
20	C022521	-	-	-	-	-	-	-
21	C022607	-	-	-	-	+	-	-
22	C020865	-	-	-	-	-	-	-
23	N006003	-	-	-	-	-	-	-
24	N013069	-	-	-	-	-	-	-
25	C020715	-	-	-	-	-	-	-
26	C021455	-	-	-	-	-	-	-
27	C021447	-	-	-	-	-	-	-
28	C022103	-	-	-	-	-	-	-
29	C022383	-	-	-	-	-	-	-
30	C022719	-	-	-	-	-	-	-
31	N009689	-	-	-	-	-	-	-
32	C024263	-	-	-	-	-	-	-
33	C024503	-	-	-	-	-	-	-
34	C019611	-	-	-	-	-	-	-
35	C023249	-	-	-	-	-	-	-
36	C024507	-	-	-	-	-	-	-
37	**N014267**	-	+	+	-	-	-	+
38	C024445	-	-	-	-	-	-	-
39	C019613	-	-	-	-	-	-	-
40	C024293	-	-	-	+	-	-	-

+ Represents inhibition of bacterial growth at the specified concentration by comparison of turbidity with a Blank assessed by the naked eye following 24 hr incubation period.

Table 1b *Antimicrobial activity of extracts numbered 41-80 on bacterial species.*

Extract ID Number		Bacterial Strains						
		A.baumani 10 µg/ml	C. difficile 5 µg/ml	C. jejuni 5 µg/ml	C. Perfringens 5 ug/ml	K. Pneumoniae 5 µg/ml	P. Aeruginosa 20 µg.ml	S. Pneumoniae 5 µg/ml
41	C022143	-	-	-	-	-	-	-
42	C022197	-	-	-	-	-	-	-
43	C022085	-	-	-	-	-	-	-
44	C022347	-	-	-	-	-	-	-
45	C022205	-	-	-	-	-	-	-
46	C022209	-	-	-	-	-	-	-
47	C023047	-	-	-	-	-	-	-
48	C022625	-	-	-	-	-	-	-
49	C022461	-	-	-	-	-	-	-
50	C024257	-	-	-	-	-	-	-
51	C022097	-	-	-	-	-	-	-
52	N011637	-	-	-	-	-	-	-
53	C023411	-	-	-	-	-	-	-
54	C021365	-	-	-	-	-	-	-
55	C023675	-	-	-	-	-	-	-
56	C022445	-	-	-	-	-	-	-
57	C021743	-	-	-	+	-	-	-
58	C024121	-	+	-	-	-	-	+
59	C022495	-	+	-	-	-	-	-
60	C024197	-	-	-	-	-	-	-
61	C024057	-	-	-	-	-	-	-
62	C025257	-	-	-	-	-	-	-
63	N112579	-	-	-	-	-	-	-
64	N109519	-	-	-	-	-	-	-
65	N11649	-	-	-	-	-	-	-
66	N111757	-	-	-	-	-	-	-
67	N109523	-	-	-	-	-	-	-
68	C024475	-	+	-	-	-	-	-
69	N112175	-	-	-	-	-	-	-
70	N109131	-	-	-	-	-	-	-
71	N111681	-	+	-	-	-	-	-
72	N111355	-	+	-	-	-	-	-
73	C024485	-	-	-	-	-	-	-
74	N113517	-	-	-	-	-	-	-
75	C021701	-	-	-	-	-	-	-
76	C021731	-	-	-	-	-	-	-
77	N111389	-	-	-	-	-	-	-
78	N111559	-	-	-	-	-	-	-
79	**C025163**	-	+	+	-	-	-	+
80	C024955	-	-	-	-	-	-	-

+ Represents inhibition of bacterial growth at the specified concentration by comparison of turbidity with a Blank assessed by the naked eye following 24 hr incubation period.

Table 1c *Antimicrobial activity of extracts numbered 81-106 on bacterial species.*

Extract ID Number		Bacterial Strains						
		A.baumani 10 µg/ml	C. difficile 5 µg/ml	C. jejuni 5 µg/ml	C. Perfringens 5 ug/ml	K. Pneumoniae 5 µg/ml	P. Aeruginosa 20 µg.ml	S. Pneumoniae 5 µg/ml
81	C022571	-	-	-	-	-	-	-
82	C024067	-	+	-	+	-	-	+
83	C022589	-	-	-	-	-	-	-
84	C024173	-	-	-	-	-	-	-
85	C023849	-	-	-	-	-	-	-
86	C024643	-	-	-	-	-	-	-
87	N050275	-	-	-	-	-	-	-
88	C024769	-	+	-	-	-	-	-
89	C021805	-	-	-	-	-	-	-
90	**C022615**	-	+	+	+	-	-	+
91	C022559	-	-	-	-	-	-	-
92	C023951	-	-	-	-	-	-	-
93	N111339	-	-	-	-	-	-	-
94	N111429	-	-	-	-	-	-	-
95	N107283	-	-	-	-	-	-	-
96	C022035	-	-	-	-	-	-	-
97	N113185	-	-	-	-	-	-	-
98	N111557	-	-	-	-	-	-	-
99	N107295	-	-	-	-	-	-	-
100	N109361	-	-	-	-	-	-	-
101	N113521	-	-	-	-	-	-	-
102	C021929	-	-	-	-	-	-	-
103	N009739	-	-	-	-	-	-	-
104	N105317	-	-	-	-	-	-	-
105	**C022687**	+	+	+	+	+	+	+
106	C024005	-	-	-	-	+	-	+

+ Represents inhibition of bacterial growth at the specified concentration by comparison of turbidity with a Blank assessed by the naked eye following 24 hr incubation period.

Discussion

The first extract chosen for its significant inhibitory activity was from *Mammea africana*. *M. africana* (Family Clusiaceae) is widely distributed in tropical Africa. The stem bark of the plant is commonly used in traditional medicine for the treatment of malaria related fever, internal heat, microbial infections, stomach pains, scabies and other skin diseases, rheumatic pain and fybromyoma [39-41]. Mammea coumarins have been isolated and identified from a variety of plant species including *Mammea africana* and other species of *Mammea*, *Mesua*, and *Calophyllum* [33]. Mammea coumarins have been found to express a wide range of bioactivities, including antibacterial [42, 43], antifungal [44], antioxidant [45], insecticidal [46], anticancer [39, 45, 47], and anti-HIV [48].

A second extract which was identified through screening was from *Phyllospongia Papyracea*. *P. Papyracea* (Family Thorectidae) is a marine sponge of the Dictyoceratida order. Two types of molecular structures have been recorded as discovered from *Phyllospongia*, scalarane sesterpenes and polybrominated biphenyl ethers [49]. While much research has been conducted on the former, the bioactivity of the latter is less well known [49]. Bromophenolic compounds are present in high concentrations in marine sponges and have been found to show antibacterial [50], antifungal [51], and anti-inflammatory activities [52] as well as cytotoxicity [25, 50, 53].

The extract from *Monanchora unguiculata* was chosen as it was shown to possess inhibitory activity on all of the bacterial species included in the screening. *M. unguiculata* (Family Crambeidae) is a marine sponge with distribution in the Indian Ocean. Investigation of the sponge *M. unguiculata* has previously led to the identification of pentacyclic guanidine alkaloids [54]. The cyclic guanidine derivatives have previously been shown to possess a broad

range of bioactivity. This class of metabolites has shown antifungal, antiviral, antiprotozoal, and antimicrobial activities. Furthermore, these compounds have also been found to exhibit cytotoxic, antimalarial, and anti-HIV activity [55, 56]. The guanidine alkaloids group includes ptilomycin A, the cramescins, crambescidins, ptilocaulins, mirabilines, and the batzelladines, which have similar structural features and biological activities. The members of this class include the pentacyclic and tricyclic guanidine alkaloids which bear the (5,6,8b)-triazaperhydroacenaphthalene skeleton [57]. Increasing interest in this group of compounds has led researchers to begin studies to synthesize such compounds [58].

The final extract that was chosen for further study was obtained from the sponge *Dysidea herbacea* (Family Dysideidae). *D. herbacea* is a tropical marine sponge which has been collected from the waters off the shore of Australia and Indonesia [59]. This sponge has two known chemotypes. The first produces sesquiterpenes and polychlorinated amino acid derivatives, while the other produces only polybrominated diphenyl ethers [60]. It has been suggested that the production of polybrominated diphenyl ethers is due to a cyanobacterium, *Oscillatoria spongeliae*, and not the sponge itself. The compounds are thought to offer a mode of protection to the sponge from predators and bacterial invasion [61]. The polybrominated diphenyl ethers have been found to exhibit a range of notable activities. They have been found to exhibit antibacterial activities on *S. aureus* and *T. Mentagrophytes*, as well as inhibition of a number of tumor-promoting enzymes [60, 62].

Previous work pertaining to these four species is far from complete. Although it is known that they all possess bioactive compounds, none of these species has previously been tested for antimicrobial activity on a broad range of bacterial pathogens.

Isolation of Active Compounds

Introduction

Natural product research often follows a number of steps intended to best utilize the biologically active compounds of plant resources. These steps are extraction, pharmacological screening, isolation and characterization of bioactive compounds, toxicological evaluation, and finally clinical evaluation. [63]. There are numerous potential setbacks involved in this type of research. Fractionation of extracts can lead to a reduction or loss of biological activity by compound break-down or loss of additive or synergistic effects between other active compounds present in the extract. Furthermore, there is no one standardized separation technique for extracts and when an active compound is isolated, it is often not possible to re-isolate even when following an identical protocol [20]. In this regard, a systematic approach to extraction and isolation of compounds was of the utmost importance in obtaining pure antimicrobial compounds.

Each extract is a complex combination of bioactive compounds and phytochemicals with different characteristics. For this reason, separation poses a challenge, often involving numerous steps and a variety of fractionation techniques [63]. Bioassay is necessary between each step to identify the active fraction/fractions. When an active fraction was identified, a separation plan was made based on the characteristics of the compounds which were deduced from the previous separation, ex. polarity, size of molecule, etc.

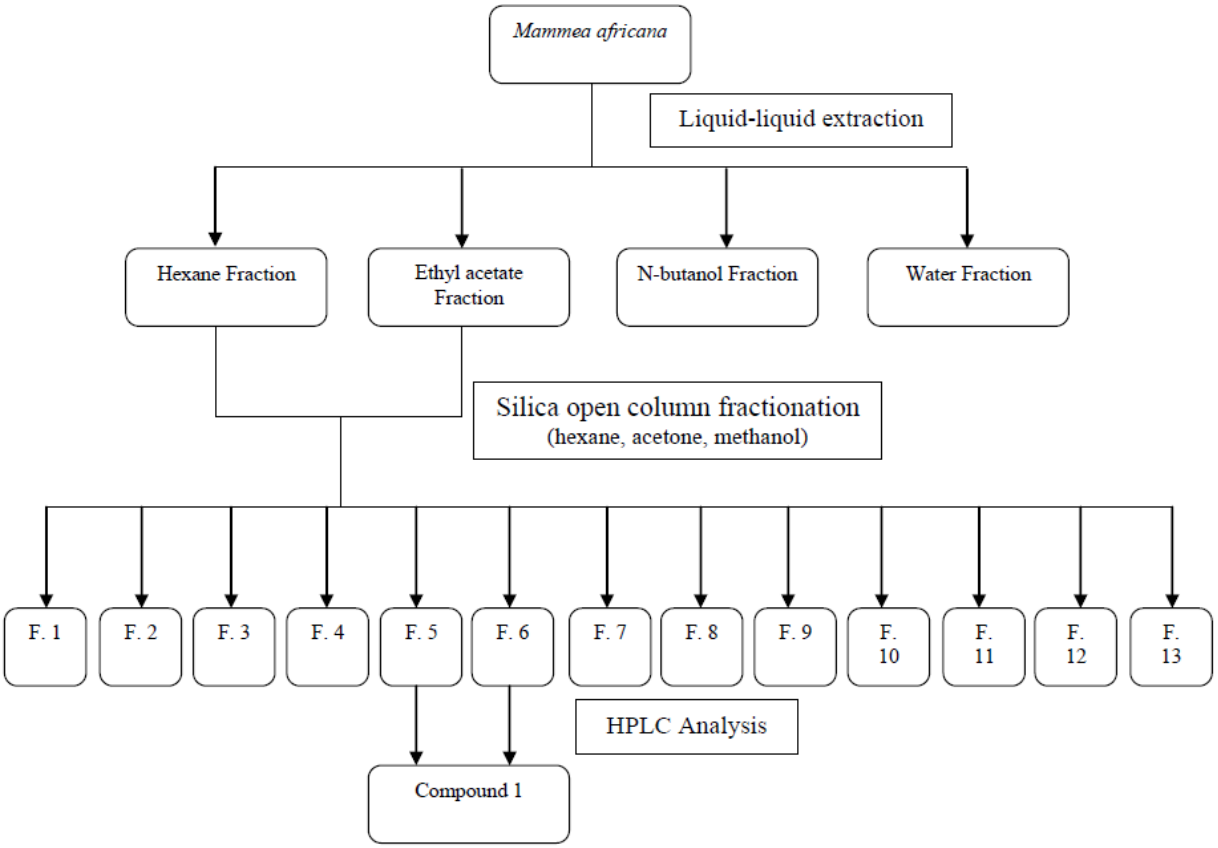
Materials and Methods

Isolation of Active Compounds - Each extract was fractionated individually as described in the following paragraphs. A combination of multiple purification techniques including liquid-liquid extraction, open column chromatography, Sephadex cartridge, high performance liquid

chromatography (HPLC) was utilized. Solvents were removed using evaporation or sublimation, to determine the final amount obtained for each sample. Samples were dissolved and diluted to 100 µg/ml in an appropriate solvent. A bioassay guided approach to fractionation was taken; in which, the activity of fractions was assessed at each stage of fractionation via the simplified microbroth dilution method previously described using a bacterial strain proven to be inhibited by the extract in the screening. Fractions showing inhibitory activity were subject to additional fractionation. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used to determine the point in the separation process when a single pure active compound has been isolated.

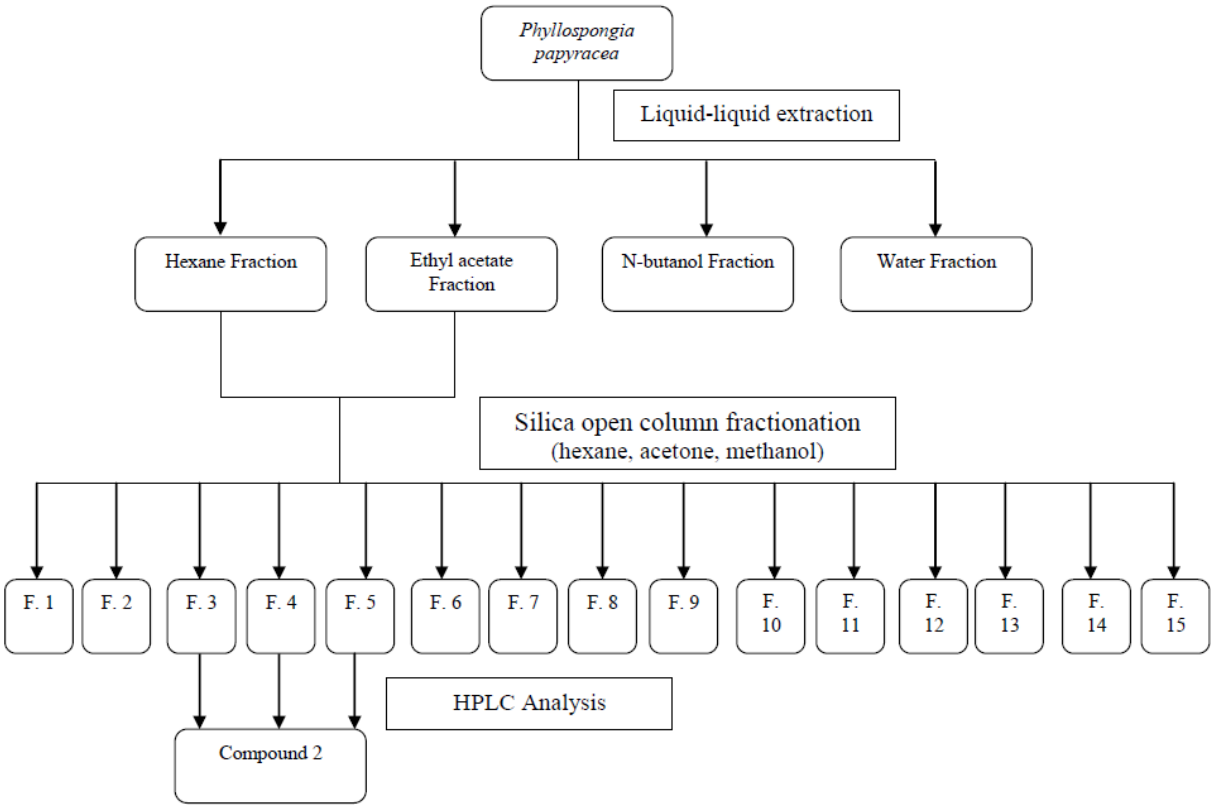
Fractionation and isolation of compound 1 from Mammea africana - Crude extract from the bark of the *M. africana* was partitioned by liquid-liquid extraction using hexane, ethyl acetate, butanol, and water. Active fractions were identified through an antibacterial activity assay. The assay revealed the hexane and ethyl acetate fractions exhibited antimicrobial activity. These two fractions were combined and subjected to fractionation via open column chromatography. The chromatography was carried out on 100 g of silica gel (40-60 µm, Acros, NJ) in a glass column (457×40 mm) and eluted with hexane, acetone, and methanol to yield a total of 13 fractions. Fraction 5 and 6 were identified as containing the active compound. HPLC with a reversed-phase C-18 column (150 x 4.6 mm) by a Hitachi DAD L-2455 detector was used to verify that a single compound was present in Fraction 5 (Scheme 1).

Scheme 1 *Flow chart depicting the bioactivity-driven fractionation of Mammea africana extract.*



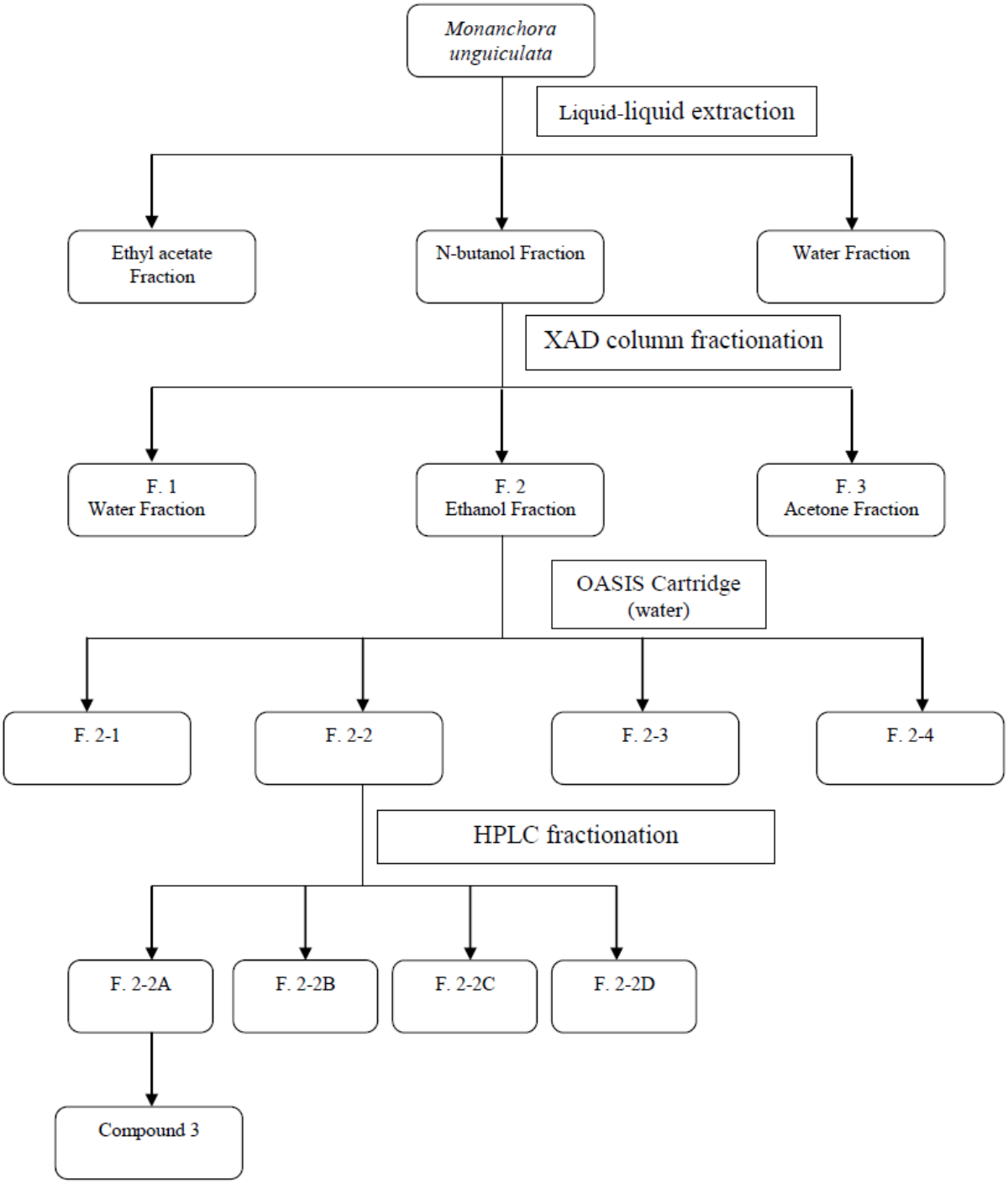
Fractionation and isolation of compound 2 from Phyllospongia papyracea - Crude extract from *P. papyracea* was partitioned by liquid-liquid extraction using hexane, ethyl acetate, n-butanol, and sterile distilled water. Bioactivity assay revealed the hexane and ethyl acetate fractions exhibited antimicrobial activity. These two fractions were combined and subjected to fractionation via open column chromatography. The chromatography was carried out on 100 g of silica gel (40-60 μm, Acros, NJ) in a glass column (457×40 mm) and eluted with hexane, acetone, and methanol to yield a total of 15 fractions. Fractions were monitored on TLC Silica Gel 60 F254 (EMD, Gibbstown, NJ) and visualized under UV (254 nm and 365 nm) and 10% H_2SO_4 (in EtOH). Fraction 3,4 and 5 were identified as active fractions and were analyzed with high performance liquid chromatography (HPLC) on a reverse phase C18 column (150 x 4.6 mm) by a Hitachi DAD L-2455 detector to determine whether a single compound was present. UV-VIS spectrum was recorded in methanol/H2O using a Hitachi DAD L-2455 (Scheme 2).

Scheme 2 *Flow chart depicting the bioactivity-driven fractionation of Phylospongia papyracea extract.*



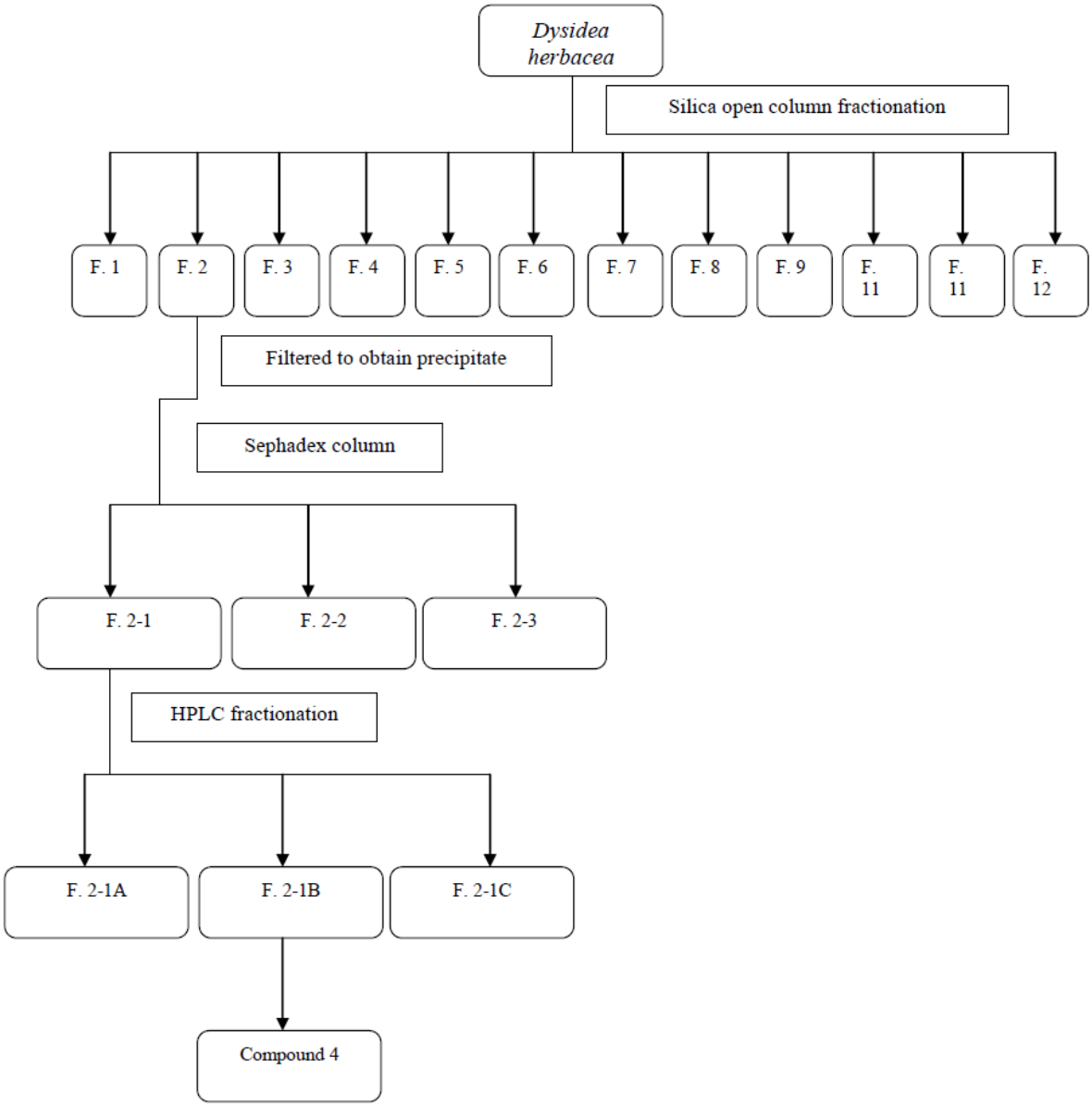
Fractionation and isolation of compound 3 from Monanchora unguiculata - Crude extract from *M. unguiculata* was partitioned by liquid-liquid extraction using ethyl acetate, butanol, and water. Active fractions were identified through an antibacterial activity assay. The assay revealed the butanol fraction exhibited antimicrobial activity. This fraction was subjected to fractionation via XAD column chromatography using the solvents water, ethanol, and finally acetone. Following bioassay, the ethanol fraction was found to contain the active compound. This fraction was further separated using OASIS cartridge chromatography and sterile distilled water as the solvent. Fraction 2-2 was found to be active, and HPLC was used to further purify the fraction using a reversed-phase C-18 column (150 x 4.6 mm) by a Hitachi DAD L-2455 detector. The first peak was collected and was analysed using HPLC to determine that it contained a purified substance (Scheme 3).

Scheme 3 *Flow chart depicting the bioactivity-driven fractionation of Monanchora unguiculata extract.*



Fractionation and isolation of compound 4 from Dysidea herbacea - Crude methanol extract from *D. herbacea* was partitioned via open column chromatography. The chromatography was carried out on 100 g of silica gel (40-60 μm, Acros, NJ) in a glass column (457×40 mm) and eluted with hexane, acetone, and methanol to yield a total of 12 fractions. Fraction 2 was identified via bioassay to contain the active fraction. It was filtered using Whatman No. 2 filter paper to separate the precipitate. The precipitate was then subjected to a Sephadex column. The first fraction to elute was active. HPLC was used to further purify the fraction using a reversed phase C-18 column (150 x 4.6 mm) by a Hitachi DAD L-2455 detector. The second peak was collected and was analysed using HPLC to determine that it contained a purified substance (Scheme 4).

Scheme 4 *Flow chart depicting the bioactivity-driven fractionation of Dysidea herbacea extract.*



Results

Extracts were fractionated individually as described following the outline depicted in Schemes 1-4. Each time a sample was fractionated; all fractions were included in a simplified microbroth dilution assay to identify the active fraction or fractions. In this way, the active fraction was purified as the inactive fractions were removed. HPLC was used to determine the presence of a single compound. If a fraction led to an HPLC chromatogram displaying a single peak, fractionation was discontinued and further identification was initiated (Figure 1-4). The chromatograms obtained through HPLC analysis of the isolated product from the four active extracts each display a prominent single peak. The final fractions, assumed to be single compounds, were named as follows: Compound 1, isolated from crude *M. africana* extract, Compound 2, isolated from crude *P. papyracea* extract, Compound 3 isolated from crude *M. unguiculata extract*, and Compound 4, isolated from crude *D. herbacea* extract.

Figure 1 *HPLC chromatogram of compound 1 isolated from Mammea africana using a 90% methanol isocratic method.*

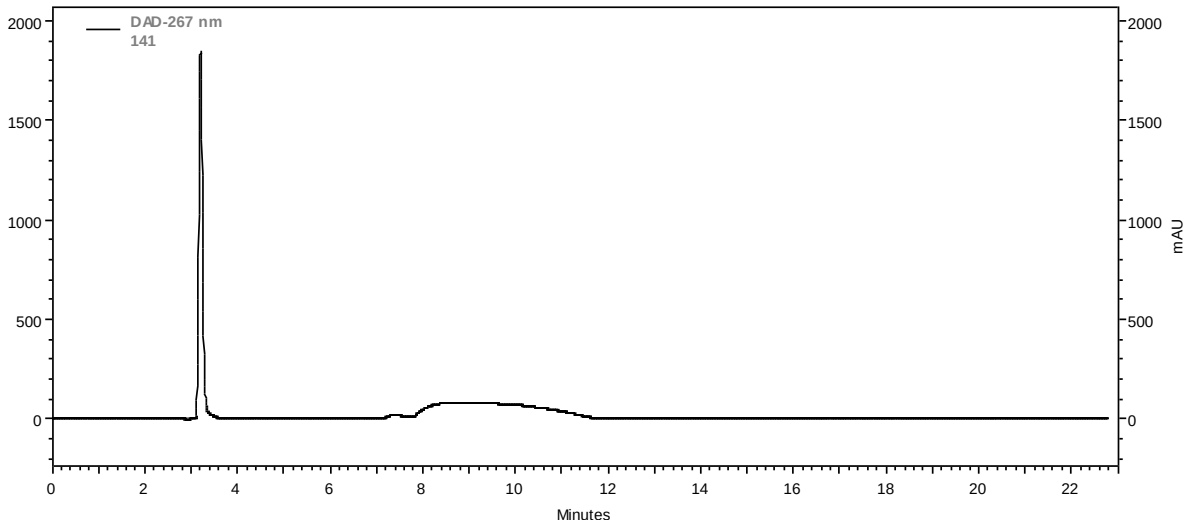


Figure 2 *HPLC chromatogram of compound 2 isolated from Phyllospongia papyracea using an isocratic 90% methanol method.*

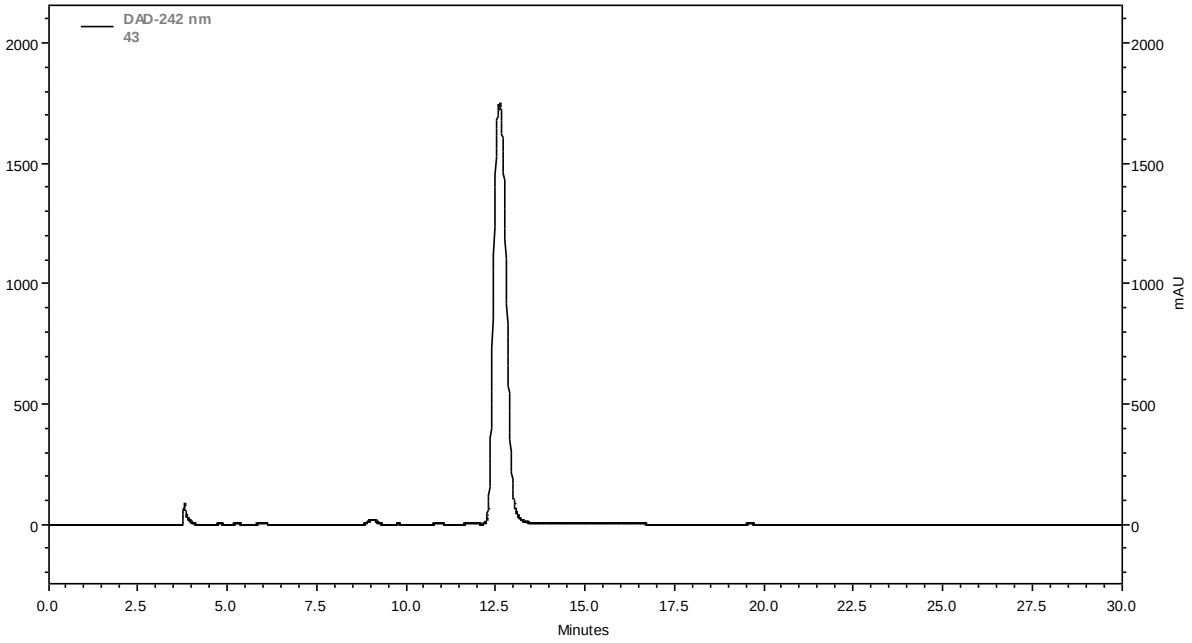


Figure 3 *HPLC Chromatogram of compound 3 isolated from Monanchora unguiculata using an isocratic 100% methanol method.*

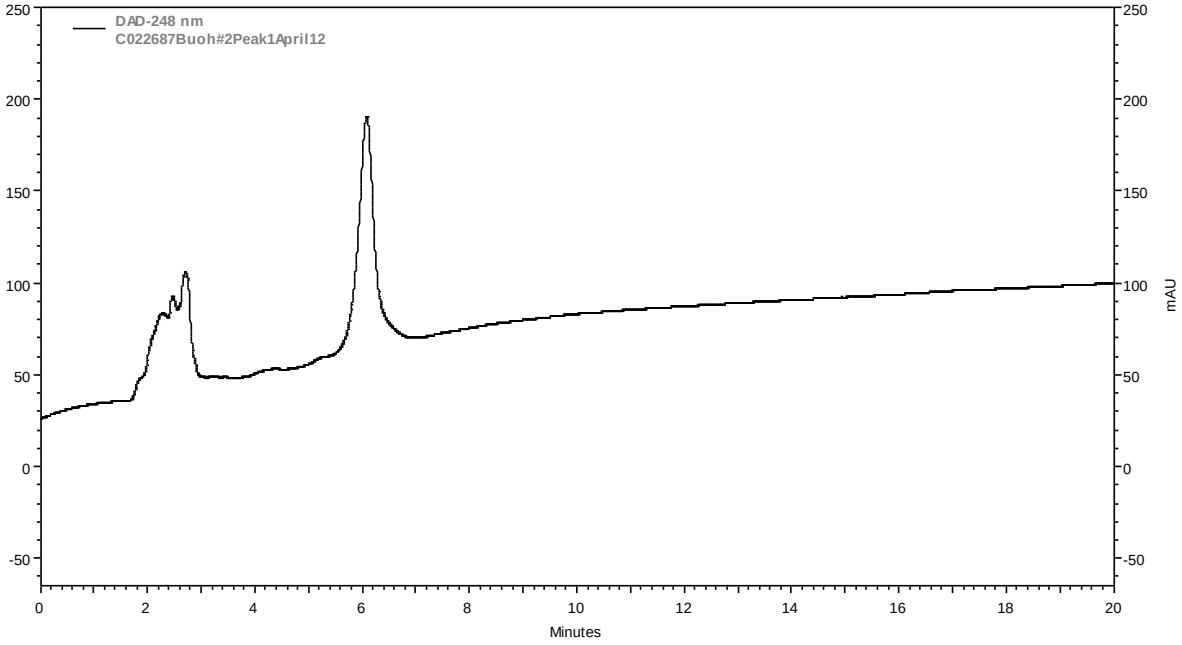
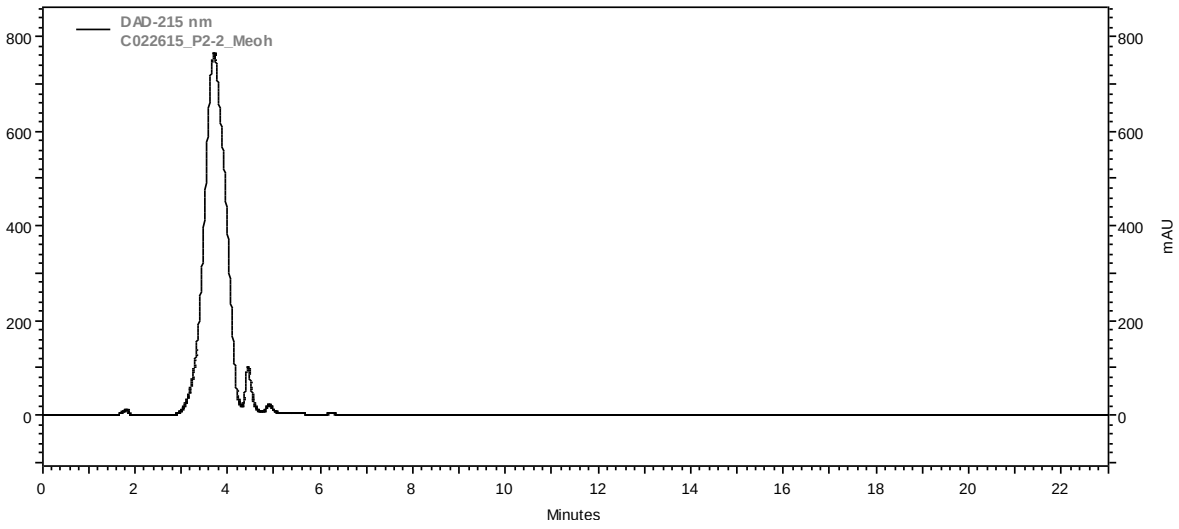


Figure 4 *HPLC chromatogram of compound 4 isolated from Dysidea herbacea using an isocratic 100% methanol method.*



Discussion

The goal of fractionation is to isolate the active compound in the extract by systematically removing the components which do not contribute to its bioactivity of interest. HPLC is often the final analytical tool used. In this case, active fractions which were believed to contain a single compound were analyzed by reversed-phase HPLC using an isocratic methanol-based method. A single peak is observed when a compound passes through the detector and absorbs UV light. When the HPLC chromatogram displayed a single peak, fractionation was discontinued and characterization studies were initiated. In this case, a single peak in an HPLC chromatogram was the signal that the active compound had been isolated. Figure 3 shows a number of peaks with retention time under 3 minutes. It is common to see a number of peaks appear on the chromatogram within the first 5 minutes and these peaks are usually associated with solvent being detected and are not necessarily attributed to multiple compounds present in the system being analyzed.

Identification of Active Compounds

Introduction

Combinatorial chemistry is often employed in determination of chemical identity and molecular formulae of unknown compounds. Confirmation relies on verification of information by comparison of liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) data. Integration of MS and NMR spectral data is the method of choice for structure verification [64].

Materials and Methods

Characterization of Active Compounds - Nuclear magnetic resonance spectroscopy (NMR) in combination with mass spectrometry (MS) was performed in the Chemistry Department at Wayne State University. Chromatograms were analyzed by the post-doctoral researcher, Dr. Sun. Through analysis of chromatograms and comparison with data previously reported in the literature, the chemical structure of the compounds was determined.

Characterization of compound 1 from Mammea africana - The compound was analyzed on a Waters LCT Premier high resolution exact mass spectrometer and Waters ZQ2000 single quadrupole mass spectrometer (Waters Corp., Milford, Massachusetts). NMR spectra (^{1}H, ^{13}C, DEPT) were recorded on a Varian Mercury 400 MHz instrument (Varian, Inc., Palo Alto, California). ^{1}H and ^{13}C were observed at 400.13 and 100.03 MHz using DMSO-*d6* as the solvent.

Characterization of compound 2 from Phyllospongia papyracea: The compound was analyzed on a Waters LCT Premier high resolution exact mass spectrometer (Waters Corp., Milford, Massachusetts). The mass spectrometer was operated in negative electrospray ionization at a temperature of 350º C and a voltage of 4 kV. Mass scan spectra were measured

from 100 m/z up to 1400 m/z at 1000 amu/second. NMR spectra (^{1}H, ^{13}C, DEPT) were recorded on a Varian Mercury 400 MHz instrument (Varian, Inc., Palo Alto, California). NMR spectra (^{1}H and ^{13}C) were observed at 400.13 and 100.03 MHz using DMSO-*d6* as the solvent. Mass spectra were obtained on a QSTAR XL MS/MS system.

Characterization of compound 3 from Monanchora unguiculata: NMR spectra were obtained with Varian VNMRS-500 MHz (500 MHz, ^{1}H;125 MHz, ^{13}C) and Agilent DD2-600 MHz (600 MHz, ^{1}H;150 MHz, ^{13}C) spectrometers; chemical shifts (δ) are reported in ppm referenced to the solvent peak. The compound was analyzed on a Waters LCT Premier high resolution exact mass spectrometer (Waters Corp., Milford, Massachusetts), EIMS on a VG 70-250-S mass spectrometer (Micromass Corp., Manchester UK) . Optical rotations were taken on a Perkin-Elmer 341 polarimeter.

Characterization of compound 4 from Dysidea herbacea: The compound was analyzed on a Waters LCT Premier high resolution exact mass spectrometer and Waters ZQ2000 single quadrupole mass spectrometer (Waters Corp., Milford, Massachusetts). NMR spectra (^{1}H, ^{13}C, DEPT) were recorded on a Varian Mercury 400 MHz instrument (Varian, Inc., Palo Alto, California). ^{1}H and ^{13}C were observed at 400.13 and 100.03 MHz using DMSO-*d6* as the solvent.

Results

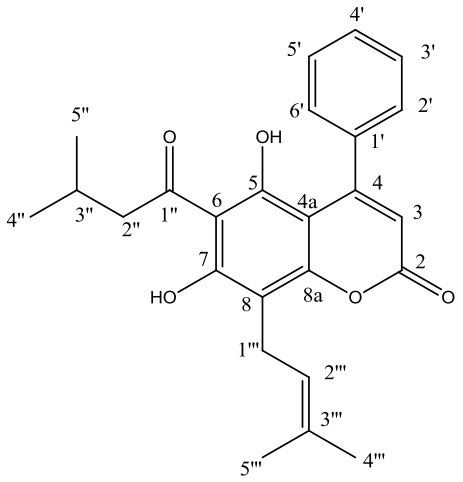
Characterization of compound 1 from Mammea africana - Fractionation of the traditional medicinal plant *Mammea africana* yielded a known coumarin with antimicrobial activity. The active compound, appearing as yellow crystals was analyzed using ^{1}H and ^{13}C NMR (Table 2). Its ^{1}H NMR spectrum showed one olefinic proton singlet at 5.93 characteristic of H-3 of a 4-substituted coumarin. ^{1}H NMR spectrum δ 7.39 (2H, dd, *J*=7.0, 3.5 Hz), 7.52 (2H,

dd, *J*=7.0, 3.5 Hz), and 7.51 (1H, t, *J*=7.0 Hz), and ^{13}C NMR spectrum 137.21, 127.39, 129.14, and 129.72 indicated the presence of one phenyl group. δ 3.55 (2H, d, *J*=8.0 Hz), 5.26 (1H, t, *J*=8.0 Hz), 1.86 (3H, s), and 1.74 (3H, s) and δ 21.65, 120.59, 135.4, 25.8, and 18.0 showed one-substituted prenyl group. δ2.85 (1H, d, *J*=8.0), 2.17 (1H, m), 0.89 (6H, d, *J*=8.0Hz) and δ207.1, 53.6, 24.7, 22.7, and 22.7 indicated the presence of one isovaleryl group. Two phenolic hydroxyl signals were at δ 11.0 and 9.9 in ^{1}H NMR, too. Comparison of its ^{1}H and ^{13}C NMR spectra with those previously reported in the literature allowed this active compound to be identified as mammea A/AA [65]. The structure of mammea A/AA is illustrated in (Figure 5).

Table 2 *^{1}H and ^{13}C NMR spectral data of compound 1 isolated from Mammea africana.*

Position	δ_C	δ_H
2	159.5 s	
3	112.7 d	5.931, s
4	154.4 s	
4a	100.8 s	
5	159.5 s	
6	107.2 s	
7	163.2 s	
8	108.0	
8a	156.6 s	
1'	137.2 s	
2', 6'	127.4d	7.39, dd, *J*=3.5, 7.0
3', 5'	129.1 d	7.52, dd, *J*=3.5, 7.0
4'	129.7 d	7.51, t, *J*=7.0
1"	207.1 s	
2"	53.6 t	2.85, d, *J*=8.0
3"	24.7 d	2.17, m
4", 5"	22.7q	0.89, d, *J*=8.0
1"'	21.7t	3.55, d, *J*=8.0
2"'	120.6d	5.26, t, *J*=8.0
3"'	135.4 s	
4"'	25.8 q	1.86, s
5"'	18.0 q	1.74, s

Figure 5 *Chemical structure of compound 1; Mammea A/AA isolated from Mammea africana*.



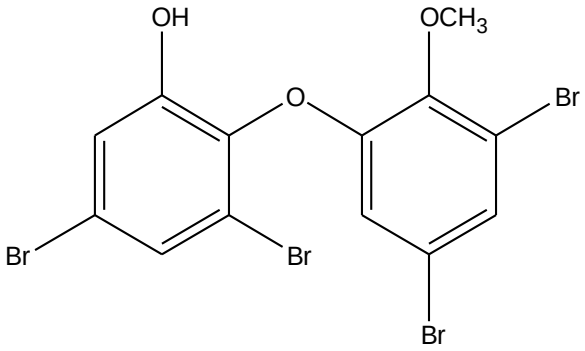
The structure of the active compound was determined to be 4-phenyl-5,7-dihydroxy-6-(3-methylbutanoyl)-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one, known as mammea A/AA.

Characterization of compound 2 isolated from Phyllospongia papyracea - Fractionation of the sponge *Pyllospongia papyracea* yielded a known tetrabrominated diphenyl ether with antimicrobial activity. The active compound, appearing as a white powder was analyzed using ^{1}H and ^{13}C NMR. The compound was identified as 4,6-Dibromo-2-(4,6-dibromo-2-hydroxyphenol) anisole (CAS80246-35-1) (Figure 10). ^{1}H NMR (CDCl3, 400 MHz) δ 7.34 (d, J= 2.4Hz, H-5′), 7.43(d, J= 2.4Hz, H-5), 7.17(d, J= 2.4Hz, H-3′), 6.77 (d, J= 2.4Hz, H-3), 4.03 (s, OCH3); ^{13}C NMR (CDCl3, 125.7 MHz) δ 150.9 (C-2), 150.4 (C-2′), 145.6 (C-1), 138.7 (C-1′), 130.4 (C-5), 127.4 (C-5'), 120.2 (C-3'), 119.9 (C-4'), 119.0 (C-6), 118.5 (C-3), 117.4 (C-4), 117.3 (C-6′), 61.6 (OCH3); ESIMS [M - H]- m/z 526.712 (15), 528.7144 (83), 530.7039 (100), 532.7091 (77), 534.7084 (20), 534.7549 (5). These spectral data were in agreement with those previously reported in the literature [53, 66].

Table 3 *^{1}H and ^{13}C NMR spectral data of compound 2 isolated from Phyllospongia papyracea.*

Position	^{1}H	^{13}C
1		145.6
2		150.9
3	6.77 (d, *J*= 2.4, H-3),	118.5
4		117.4
5	7.43(d, *J*= 2.4, H-5),	130.4
6		119.0
1′		138.7
2′		150.4
3′	7.17(d, *J*= 2.4, H-3′),	120.2
4′		119.9
5′	7.34 (d, *J*= 2.4, H-5′),	127.4
6′		117.3
OCH3	4.03 (s)	61.6

Figure 6 *Chemical structure of compound 2; a tetrabominated diphenyl ether isolated from Phyllospongia papyracea.*



The structure of the active compound was determined to be Phenol, 3,5-dibromo-2-(3,5-dibromo-2-methoxyphenoxy).

Characterization of compound 3 isolated from Monanchora unguiculata – Semi-preparative HPLC was used to separate chromatographic peaks. One sample collected was found to exhibit antimicrobial activity. The sample appeared as a light yellow glue. Although this appeared as a single chromatographic peak, and was believed to be a pure compound, compound 3 actually contains a mixture of compounds. Through comparison of ^{1}H and ^{13}C NMR data with those reported in the literature regarding similar marine species, it was found that the sample exhibited the same 'vessel' part system and relative stereochemistry as crambescidin 800, ptilomycalin A, and fromianmycalin [67-70]. Further analysis of ^{1}H, ^{13}C NMR data established that a spermidine residue was present, as the 'anchor' however, the exact structure could not be differentiated. By analysis of DEPT, COSY, TOCSY, HSQC, and HMBC, one of spermidine residues was the same as fromianmycalin [69]; one of as crambescidin 800 [67]; another as ptilomycalin A [67, 68], however, the hydroxyl group could not be assigned.

The compound mixture appeared as a light yellow oil, $[\alpha]_D^{20}$ 0° (*c* 0.21, MeOH); UV (MeOH/water) λ_{max} 248nm; HRESIMS *m/z* 801.6217 $[M+H]^+$ 401.8152 [M+2]/2 (calcd for $C_{45}H_{81}N_6O_6$, 801.6218, monanchocidin F and crambescidin 800); 392.8095 [M+2]/2 (calcd for $C_{45}H_{79}N_6O_5$, 783.6112, fromianmycalin) ; EIMS *m/z* 782.9 (1.2), 709.8 (2.2), 683.8 (4.3), 643 (3.3), 613.7 (2.8), 574.7 (6.6), 544.6 (4.8), 503.3 (8.2), 429.3 (14.9), 402.4 (11.3), 360.4 (12.8), 359.4 (23.5), 358.4 (23.7), 330.4 (27.3), 312.4 (24.7), 311.5 (24.2), 288.3 (23.9), 262.3 (31.1), 261.3 (26.6), 192.2 (33.1), 191.2 (33.7), 149.2 (29.6),113.2 (25.1), 111.2 (25.8), 98.2 (31.1), 99.2 (28.2), 36.0 (100).

In HRESIMS spectrum, a pseudomolecular ion, [M+H], at m/z 801.6217, and two [M+2]/2 ion peak groups at m/z 401.3131, 401.8152, 402.3191, and 402.8218; 392.3080, 392.8095, 393.3137, and 393.8580, were assigned the molecular formula $C_{45}H_{80}N_6O_6$ and

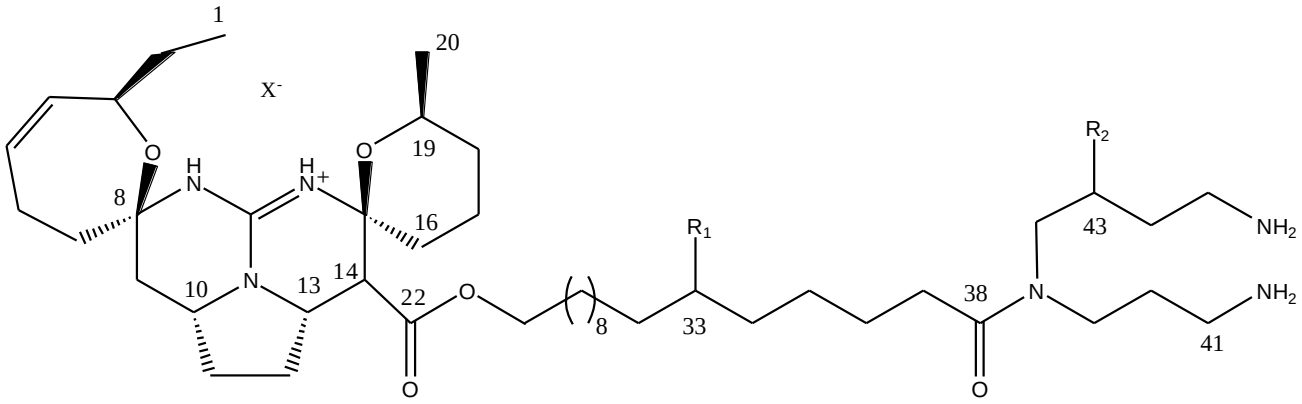
$C_{45}H_{79}N_6O_5$, respectively. EIMS data presented the fragmentation ion at m/z 729 (M-71) and 613 (M-187) from M ion (m/z 800), corresponding to the losses of C_4H_9N and $C_9H_{21}N_3O$, respectively, which showed the existence of ptilomycalin A's spermidine residue again. The position of hydroxyl group was assigned at C-33 depending on the one pair of ion at m/z 574/326 and 544/356 (scheme 5). The configuration of C-33 can not be determined based on the results of the application of the Mosher's method because Δδ of C-32 and 34 showed the same positive value. And the optical value was 0°. This was identified as one new compound, a polycyclic guanidine alkaloid, monanchocidin F (Figure 7), the NMR data were assigned as Table 3 dependent on 2D NMR as well as two known compounds crambescidin 800 and fromianmycalin (Figure 8).

Tabel 4. *^{1}H and ^{13}C NMR data for compound mixture in compound 3 (in CD_3OD,)*

	Monanchocidin F		Crambescidin 800		Fromianmycalin	
	^{1}H NMR	^{1}H NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	0.86, t, 7.8Hz	0.86, t, 7.8Hz	0.86, t, 7.8Hz	9.42	0.86, t, 7.8Hz	9.42
2a	1.45, m	1.45, m	1.45, m	26.17	1.45, m	26.17
2b	1.51, m	1.51, m	1.51, m		1.51, m	
3	4.40, br d, 12.0 Hz	4.40, br d, 12.0 Hz	4.40, br d, 12.0 Hz	72.3	4.40, br d, 12.0 Hz	72.3
4	5.49, br d, 12.0 Hz	5.49, br d, 12.0 Hz	5.49, br d, 12.0 Hz	132.93	5.49, br d, 12.0 Hz	132.93
5	5.68, br t, 12.0 Hz	5.68, br t, 12.0 Hz	5.68, br t, 12.0 Hz	129.88	5.68, br t, 12.0 Hz	129.88
6a	2.13, m	2.13, m	2.13, m	23.01	2.13, m	23.01
6b	2.35, m	2.35, m	2.35, m		2.35, m	
7a	1.94, m	1.94, m	1.94, m	38.17	1.94, m	38.17
7b	2.38, m	2.38, m	2.38, m		2.38, m	
8				83.64		83.64
9a	1.40, t, 12.0 Hz	1.40, t, 12.0 Hz	1.40, t, 12.0 Hz	37.86	1.40, t, 12.0 Hz	37.86
9b	2.62, dd, 15.6, 6.0 Hz	2.62, dd, 15.6, 6.0 Hz	2.62, dd, 15.6, 6.0 Hz		2.62, dd, 15.6, 6.0 Hz	
10	4.04, m	4.04, m	4.04, m	54.10	4.04, m	54.10
11a	1.68, m	1.68, m	1.68, m	30.12	1.68, m	30.12
11b	2.24, m	2.24, m	2.24, m		2.24, m	
12a	1.65, m	1.65, m	1.65, m	28.19	1.65, m	28.19
12b	2.26, m	2.26, m	2.26, m		2.26, m	
13	4.33, m	4.33, m	4.33, m	52.55	4.33, m	52.55
14	3.06, d, 6.0	3.06, d, 6.0	3.06, d, 6.0	49.34	3.06, d, 6.0	49.34
15				80.68		80.68
16a	1.52, m	1.52, m	1.52, m	32.73	1.52, m	32.73
16b	1.80, m	1.80, m	1.80, m		1.80, m	
17a	1.79, m	1.79, m	1.79, m	18.04	1.79, m	18.04
17b	1.91, m	1.91, m	1.91, m		1.91, m	
18a	1.75, m	1.75, m	1.75, m	32.55	1.75, m	32.55
18b	1.92, m	1.92, m	1.92, m		1.92, m	
19	3.84, br dd, 12.6, 6.0 Hz	3.84, br dd, 12.6, 6.0 Hz	3.84, br dd, 12.6, 6.0 Hz	68.40	3.84, br dd, 12.6, 6.0 Hz	68.40
20	1.07, d, 7.2 Hz	1.07, d, 7.2 Hz	1.07, d, 7.2 Hz	20.40	1.07, d, 7.2 Hz	20.40
21				150.1		150.1
22				168.71		168.71
23	4.11, t, 7.2 Hz	4.11, t, 7.2 Hz	4.13, t, 7.2 Hz	68.55	4.11, t, 7.2 Hz	68.55
24	1.63, m	1.63, m	1.97	39.93	1.63, m	39.93
25	1.29, m	1.29, m	4.03	67.04	1.29, m	67.04
26	1.29, m	1.29, m	1.41	37.86	1.29, m	37.86
27	1.29, m	1.29, m	1.29, m	28.84-30.12	1.29, m	28.84-30.12
28-31	1.29, m	1.29, m	1.29, m	28.84-30.12	1.29, m	28.84-30.12
32	1.69	1.29, m	1.29, m	28.84-30.12	1.29, m	28.84-30.12
33	4.03, m	1.29, m	1.29, m	28.84-30.12	1.29, m	28.84-30.12
34	1.40	1.29, m	1.29, m	28.84-30.12	1.29, m	28.84-30.12
35	1.29	1.29, m	1.29, m	28.84-30.12	1.29, m	28.84-30.12
36	1.80, m	1.80, m	1.80, m	27.67	1.80, m	27.67
37	2.48, m	2.48, m	2.48, m	32.76	2.48, m	32.76
38				166.36		166.36

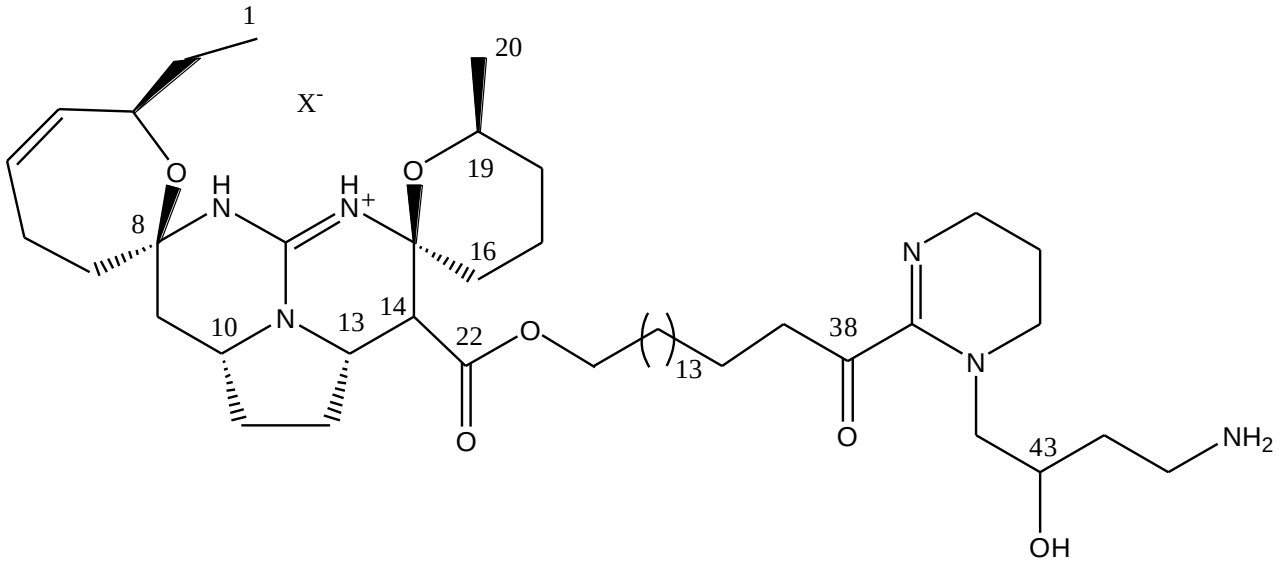
39a	3.37, m	3.38, m	3.38, m	47.84	3.38, m	47.84
39b	3.62, m	3.64, m	3.56, m		3.64, m	
40	1.92, m	1.92, m	2.07, m	19.86	1.92, m	19.86
41	2.88, m	2.88, m	3.38, m	39.94	2.88, m	39.94
			3.53, m			
42a	3.51, m	3.32	3.41, m	58.51	3.32	58.51
42b	3.63, m	3.46	3.69, m		3.46	
43a	1.95 m	3.95, m	4.03, m	69.38	3.95, m	69.38
43b	2.05 m					
44	1.65, m	1.75	1.75	32.87	1.75	32.87
	1.75, m	1.93	1.93		1.93	
45	3.12, m	3.12, m	3.12, m	38.52	3.12, m	38.52

Figure 7 *Chemical structure of a component of compound 3; monanchocidin F isolated from Monanchora unguiculata*



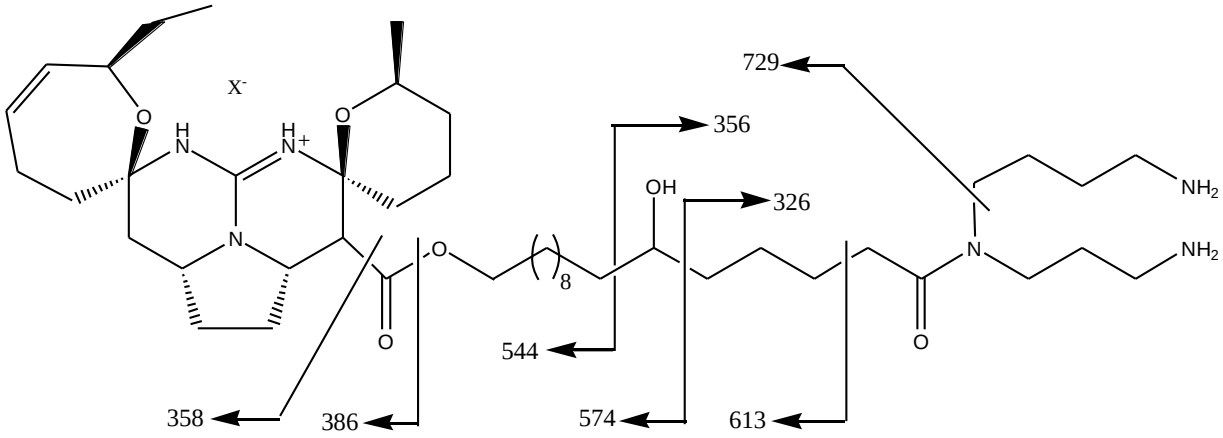
The mixture of compounds was determined to include the new compound monanchocidin F, where R_1=OH, R_2=H.

Figure 8 *Chemical structure of components of compound 3; the compounds cambescidin and fromianmycalin, isolated from Monanchora unguiculata.*



The mixture of compounds was determined to include the known compounds crambescidin 800, where R_1=H, and fromainmycalin, where R_2= OH.

Scheme 5 *Characterization of new compound monanchocidin F by MS fragmentation (m/z values).*

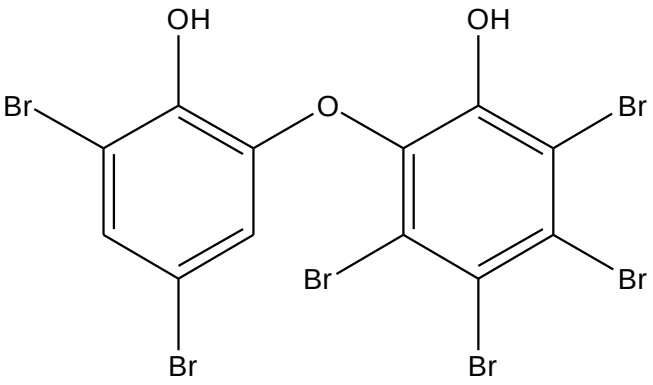


Characterization of compound 4 isolated from Dysidea herbacea – Fractionation of *Dysidea herbacea* extract led to the isolation of a single known compound. The compound appeared as a light yellow glu. ^{1}H NMR (methanol-d_4, 500 MHz) δ 7.32 (1H, d, J=2.5 Hz, H-4′), 6.43 (1H, d, J=2.5 Hz, H-6′); ^{13}C NMR (methanol-d_4, 125.7 MHz) δ 150.3 (C-1), 146.6 (C-1′), 145.1 (C-2′), 141.1 (C-2), 130.1 (C-4′), 126.7 (C-3), 121.3 (C-4), 118.9 (C-5), 116.8 (C-6), 116.3 (C-6′), 112.2 (C-3′), 111.2 (C-5′); ESIMS $[M-H]^-$ m/z 668.91 (2), 670.86 (24), 672.89 (74), 674.87 (100), 676.85 (67), 678.88 (22), 680.8 7(2); ESIMS $[M+Na]^+$ m/z 691.92 (3), 694.89 (24), 696.85 (65), 698.85 (100), 700.85 (58), 702.83 (19), 705.13 (3). Through comparison of spectral data previously reported in the literature, the compound was determined to be the tetrabrominated diphenyl ether: 2-(3′,5′-dibromo-2′-hydroxyphenoxy)-3,4,5,6-tetrabromophenol [71-73] (Table 5).

Table 5 *^{1}H and ^{13}C NMR spectral data of compound 2 isolated from Dysidea herbacea.*

Position	^{1}H	^{13}C
1		150.3
2		141.1
3		126.7
4		121.3
5		118.9
6		116.8
1′		146.6
2′		145.1
3′		112.2
4′	7.32 (1H, d, *J*=2.5)	130.1
5′		111.2
6′	6.43 (1H, d, *J*=2.5)	116.3

Figure 9 *Chemical structure of compound 4; an oxy-polyhalogenated diphenyl ether isolated from Dysidea herbacea.*



The structure of the active compound was determined to be 2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-3,4,5,6-tetrabromophenol.

Discussion

The activity-driven isolation of the *M. africana* extract led to the identification of an active component, mammea A/AA. Mammea A/AA is a 4-phenylcoumarin that has shown *in vitro* anti-proliferative activity on a variety of cancer cells [39, 45, 74, 75], high antioxidant activity [45], and low cytotoxicity to normal cells [75-77], suggesting that mammea A/AA possesses unique bioactivities worth further investigation. The compound has also been found to inhibit the growth of methicillin resistant *Staphylococcus aureus* (MRSA), and methicillin susceptible *Staphylococcus aureus* (MSSA), as well as *Enterococcus faecalis* bacteria [39, 42, 43]. The compound mammea A/AA was also previously tested for *in vitro* susceptibility against strains of *Escherichia coli*, *Enterobacter cloacae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Acinetobacter spp.*, as well as *Candida albicans* and *Cryptococcus neoformans*. Mammea A/AB combined with mammea A/AA in the ratio 4:1 was proven to be ineffective as an antimicrobial against these gram-negative bacteria and fungi demonstrating MICs above 128 µg/ml [42].

Mammea A/AA has previously been studied by a number of research groups in an effort to evaluate its anti-cancer activity. This compound was found to exhibit cytotoxicity against the human 9-KB cancer cell [39]. Researchers also found mammea A/AA to exhibit cytotoxic activities in the SW-480, HT-29, and HCT-116 human colon cancer cell lines at concentrations comparable to 5-fluorouracil, a drug frequently used for human colon cancer treatment [45]. An IC_{50} value of 6.4 µg/ml for mammea A/AA cyclo has been reported for its cytotoxicity against A2780 ovarian cancer cells [74]. The cytotoxicity of mammea A/AA was assayed using four cancer cell lines (HeLa, Calo, SW480, and SW620) and the normal peripheral-blood mononuclear cells. Results proved that mammea A/AA induced an apoptosis-like cell death in all

tested cancer cell lines, while showing very low cytotoxicity levels in normal peripheral-blood mononuclear cells [75]. Furthermore, an investigation of the cytotoxicity of mammea A/AA on normal human lymphocyte cultures, found that mammea A/AA did not show toxicity, and demonstrated an LC_{100} of 120.5 µg/ml [76]. Similarly, this compound was found to be non-toxic at 50 µM [77]. Support for the further development of mammea A/AA and other mammea coumarins is prevalent among researchers studying this class of compounds, which are thought to have significant potential as an alternative antimicrobial in the prevention and treatment of human infections, and applications as a promising candidate for anticancer treatment.

Bioactivity-guided isolation of the *P. papyracea* extract led to the identification of the active component, 4,6-Dibromo-2-(4,6-dibromo-2-hydroxyphenol) anisole [53]. This compound was previously tested for its antimicrobial activity and was found to possess antimicrobial activity by inhibiting *S. aureus* and *T. mentographytes* (MIC_{100} 0.15, 1.56 µg/ml, respectively) [50]. Furthermore, it has been found to posess antimicroalgal activity against *S. costatum* and *B. submaria* at 0.5 p.p.m. [53]. A compound with a structure later revised to match this compound was found to inhibit hypoxia-induced HIF-1 activation (IC_{50}=4.3µM), but also reduced the viability of T47D and MDA-MD-231 breast tumor cells [4].

The fractionation of a *M. unguiculata* extract led to isolation of a group of polycyclic guanidine alkaloids. Similar compounds have previously been isolated from the marine sponge *Monanchora pulcha*. The known compounds which were idenitfied as being present were crambescidin 800, fromainmycalin, and ptilomycalin A. An unknown compound was identified as Monanchocidin F. The first identified monanchocidin compound, monachocidin A was revealed as exhibiting a number of uncommon features [57]. Recently, the monanchocidins B-E were revealed which contain the same structural features; the polycyclic moiety previously

referred to as the “vessel”, the vicinal hemiketal in a substituted morpholinone ring “anchor” and hydrocarbon “chain”[78]. The monanchocidin group has previously identified as exhibiting cytotoxicity against a number of human cell lines, including human leukemia THP-1 (IC_{50} 5.1 µM), and human cervix epitheliod carcinoma Hela (IC_{50} 11.8 µM). This compound was also found to inhibit mouse epidermal JB6 C141 (IC50 12.3 µM) cell lines [57].

Bioactivity-driven isolation of the antimicrobial component of the final extract, *D. herbacea* led to the compound 2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-3,4,5,6-tetrabromophenol. The oxy-polyhalogenated diphenyl ether group of compounds has previously been found to display inhibitory activity against the bacteria *Bacillus subtilis* and fungus *Cladosporium cucumerinum* [61]. Their range of activities also includes anti-inflammatory properties, lipoxygenase inhibition, and anticancer activity [79, 80]. The oxy-polyhalogenated diphenyl ether group has been investigated for the compounds’ ability to inhibit the overexpression of Mcl-1 in cancerous cells and it was concluded that these compounds may take advantage of a synergistic or additive effect, where combined compounds exhibit activities greater than a single isolated oxy-halogenated diphenyl ether [73].

CHAPTER 3: CHARACTERIZATION OF ANTIMOCROBIAL ACTIVITY OF ISOLATED COMPOUNDS

Minimum Inhibitory Concentration (MIC)

Introduction

Broth microdilution is the most popular method for performing antimicrobial susceptibility testing in the U.S. One of the reasons for its popularity is that it produces results comparable to the "gold standard": the agar dilution method. Furthermore this method is useful when testing a number of isolates at a number of different concentrations [81]. The protocol for this method is available in the Clinical and Laboratory Institutes (CLSI) document M7-A8 and M11-A8 [37, 38]. The lowest concentration of an antibiotic that will inhibit the growth of the organism being tested is referred to as the minimal inhibitory concentration (MIC). This value is usually expressed in µg/ml and is useful to clinicians to determine the appropriate antibacterial agent and dosage to be used to treat a patient [82]. Antibiotic agents are included in assays for a variety of reasons. An antibiotic known to inhibit the bacterial species being tested must be included as a quality control. An antibiotic agent that is currently used to treat the bacterial infection must also be included in the assays in order for researchers to compare the efficacy of the natural antimicrobial with that of pharmaceutical agents. Finally, antibiotics may be included in the tests to determine susceptibility of a bacterial species to the antibiotic.

Materials and Methods

The bacterial species included in MIC determination testing were: *A. baumanii*, *Bacillus subtilis;* ATCC 6051, *C. jejuni*, *Escherichia coli 0157 H:7*; ATCC 25922, *Listeria monocytogenes*; ATCC 23074, *K. pneumoniae*, *P. aeruginosa*, *Salmonella enterica* serovar thyphimurium; ATCC 19585, *Staphylococcus areus*; NCTC 8325 and *S. pneumoniae.* The

original plant extract and the active compound were dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution with the concentration of 1 mg/ml. This was further diluted in sterile distilled water to prepare test solutions with the concentration of 100 µg/ml. MIC evaluations of aerobic and anaerobic bacteria were determined by the broth microdilution method according to CLSI guidelines [37, 38]. MIC evaluations of microaerophilic bacteria were determined as previously described [83]. In brief, bacterial colonies were selected from 24 h cultures, suspended in sterile distilled water and adjusted to match a 0.5 McFarland standard by measuring absorbance at 625 nm using an HTS 7000 Bio Assay Reader (Perkin Elmer, Norwalk, CT). The bacterial cell numbers were adjusted to approximately 1 x 10^5 colony forming units (CFU)/ml in CAMHB, BHI broth, or nutrient broth. Plant extracts, isolated active compounds, and antibiotics (100 µg/ml) were subjected to serial two-fold dilution in a 96-well microtiter plate. Final test concentrations ranged from .0125 to 16 µg/ml. Assay plates were incubated in appropriate conditions (aerobic, anaerobic, or microaerophilic) at 37 ºC for 24 h, and growth was assessed by measuring absorbance at 625 nm using the Bio Assay Reader. MIC's of antibiotic agents was interpreted as the lowest concentration of antibiotic agent to cause an 80% or greater reduction of growth compared to the growth control. The growth control consisted of DMSO diluted in sterile distilled water (1:9), and broth, but no test samples. A blank containing no bacteria was also included. Stock solution of antibiotics, erythromycin, carbenicillin, ciprofloxacin, cefoxitin, fusidic acid, imipenem, levofloxacin, moxifloxacin, rifampicin, tetracycline, and vancomycin were prepared according to manufacturer's suggestions. These were diluted to 100 µg/ml in sterile distilled water and included in the assay. The assays were performed three times and MIC values were reported as the mean value of each assay.

Results

Compound 1 isolated from *M. africana* displayed inhibitory activity on *A. baumannii* at 2 µg/ml, while the original *M. africana* extract did not exhibit inhibitory activity on this bacterial species even at 16 µg/ml. Furthermore, compound 1 inhibited the growth of the bacteria *B. subtilis*, *C. jejuni*, *L. monocytogenes*, *P. aeruginosa*, *S. aureus*, and *S. Pneumoniae* with MIC 0.5 µg/ml. Compound 1 did not inhibit *E. coli* O157:H7, *K. Pneumoniae*, or *S. Typhimurium* at concentrations up to 16 µg/ml as displayed in Table 6.

Compound 2 isolated from *P. Papyracea* displayed inhibitory activity against *B. subtilis* at a concentration of 1 µg/ml, *C. jejuni* at 2 µg/ml, *L. monocytogenes* at 8 µg/ml, *P. aeruginosa* at 4 µg/ml, *S. aureus* at 1 µg/ml, and *S. pneumoniae* at 1 µg/ml (Table 7). Compared with the original extract, the isolated compound displayed a reduced bioactivity, with the isolated compound displaying MIC's at levels between 1 and 4-fold higher than the original extract. Compound 2 did not show inhibition of growth for the bacteria *A. baumannii*, *E. coli* 0157:H7, *K. pneumoniae*, or *S. typhimurium* at concentrations up to 16 µg/ml.

The mixture of compounds in the fraction known as compound 3 displayed antimicrobial activity against *S. aureus* at 0.25 µg/ml, *B. subtilis* at 0.5 µg/ml, *C. Jejuni*, *L. monocytogenes*, *P. aeruginosa*, and *S. pneumoniae* at 2 µg/ml, and *E. coli* 0157:H7, *K. pneumoniae*, and *S. typhimurium* at 4 µg/ml (Table 8). This mixture of compounds was found to be an excellent inhibitor of every species of bacteria included in the experiment. The mixture of compounds displayed activity comparable to the original extract. MIC's for the mixture of compounds was equal to or within 2-fold higher or lower than the MIC of the original extract. Furthermore, compound 3 was the only one of the 4 compounds which inhibited the bacterial species, *E. coli* 0157:H7, *K. pneumoniae*, and *S. typhimurium*.

Compound 4, isolated from *D. herbacea*, displayed inhibitory activity on *B. subtilis* and *S. pneumoniae* at 0.5 µg/ml, *C. Jejuni* and *P. aeuruginosa* at 0.25 µg/ml, *L. monocytogenes* at 2 µg/ml, and *S. aureus* at 0.125 µg/ml (Table 9). The isolated compound displayed MIC's at or below the MIC of the original extract. This compound failed to inhibit the bacterial species *A. baumannii*, *E. coli*, *K. pneumoniae*, and *S. typhimurium* at concentrations up to 16 µg/ml.

MIC's of commonly used antibiotics against the tested bacterial strains were also included for comparison. Imipinem, a pharmaceutical agent displayed an MIC at 0.125 µg/ml against *A. Baumannii* (Table 10). *B. subtilis* was inhibited by the antibiotic agents ciprofloxacin, carbenicillin and tetracycline at 0.25 µg/ml, 0.125 µg/ml and 0.5 µg/ml, respectively. *C. jejuni* was inhibited by moxifloxacin at 0.125 µg/ml, ciprofloxacin and levofloxacin at 0.25 µg/ml and erythromycin at 0.5 µg/ml. *E. coli* was inhibited by the antibiotics moxifloxacin at 0.125 µg/ml, and ciprofloxacin and levofloxacin at 0.25 µg/ml. However, erythromycin and vancomycin did not inhibit *E.coli* bacteria at concentrations up to 16 µ/ml. *L. Monocytogenes* was inhibited by erythromycin at 0.25 µg/ml, tetracycline and vancomycin at 0.5 µg/ml and carbenicillin at 8 µg/ml. *K. pneumoniae* was inhibited by ciprofloxacin at 0.125 µg/ml and at 0.25 µg/ml by cefoxitin and imipenem. *P. aeruginosa* was inhibited by carbenicillin at 0.25 µg/ml and ciprofloxacin and imipenem at 0.125 µg/ml. MIC of the antibiotics ciprofloxacin and imipenem on *S. typhimurium* were 0.125 and 1 µg/ml, respectively. *S. pneumoniae* was inhibited by ciprofloxacin, levofloxacin, and moxifloxacin at 0.125 µg/ml. Finally, *S. aureus* was inhibited by the antibiotic agents fusidic acid and rifampicin at 0.125 µg/ml and carbenicillin and erythromycin at 0.5 µg.ml.

Table 6 *The minimum inhibitory concentration (µg/ml) of bacterial growth of bacterial strains exerted by Mammea africana plant crude extract and the isolated coumarin mammea A/AA; compound 1.*

	MIC (µg/ml)*	
	Crude *Mammea Africana* Extract	Compound 1
Acinetobacter baumannii	>16	2
Bacillus subtilis	0.5	0.5
Campylobacter jejuni	2.0	0.5
Escherichia coli 0157H7	>16	>16
Listeria monocytogenes	0.5	0.5
Klebsiella pneumoniae	>16	>16
Pseudomonas aeruginosa	1	0.5
Salmonella typhimurium	>16	>16
Stapylococcus aureus	0.5	0.5
Streptococcus pneumoniae	0.25	0.5

* Each value represents the mean of three observations.

Table 7 *The minimum inhibitory concentration (µg/ml) of bacterial growth of bacterial strains exerted by Phyllospongia papyracea plant crude extract and the isolated tetrabrominated diphenyl ether; compound 2.*

	MIC (µg/ml)*	
	Crude *Phyllospongia papyracea* Extract	Compound 2
Acinetobacter baumannii	>16	>16
Bacillus subtilis	0.5	1
Campylobacter Jejuni	1	2
Escherichia coli 0157 H:7	>16	>16
Listeria monocytogenes	1	8
Klebsiella pneumoniae	>16	>16
Pseudomonas aeruginosa	0.25	4
Salmonella typhimurium	>16	>16
Stapylococcus aureus	0.125	1
Streptococcus pneumoniae	1	4

* Each value represents the mean of three observations.

Table 8 *The minimum inhibitory concentration (µg/ml) of bacterial growth of bacterial strains exerted by Monanchora unguiculata plant crude extract and the isolated active mixture of compounds; compound 3.*

	MIC (µg/ml)*	
	Crude *Monanchora unguiculata* Extract	Compound 3
Acinetobacter baumannii	4	4
Bacillus subtilis	2	0.5
Campylobacter jejuni	0.25	2
Escherichia coli 0157 H:7	4	4
Listeria monocytogenes	4	2
Klebsiella pneumoniae	4	4
Pseudomonas aeruginosa	1	2
Salmonella typhimurium	2	4
Staphylococcus aureus	1	0.25
Streptococcus pneumoniae	0.5	2

* Each value represents the mean of three observations.

Table 9 *The minimum inhibitory concentration (µg/ml) of bacterial growth of bacterial strains exerted by Dysidea herbacea plant crude extract and the isolated active compound; compound 4.*

	MIC (µg/ml)*	
	Crude *Dysidea herbacea* Extract	Compound 4
Acinetobacter baumannii	>16	>16
Bacillus subtilis	1	0.5
Campylobacter jejuni	1.0	0.25
Escherichia coli 0157 H:7	>16	>16
Listeria monocytogenes	2	2
Klebsiella pneumonia	>16	>16
Pseudomonas aeruginosa	0.25	0.25
Salmonella typhimurium	>16	>16
Stapylococcus aureus	0.5	0.125
Streptococcus pneumoniae	0.5	0.5

* Each value represents the mean of three observations.

Table 10 *The minimum inhibitory concentration (μg/ml) on bacterial growth of bacterial strains exerted by commonly used antibiotics.*

	Antibiotic MIC (μg/ml)*											
	Erythromycin	Carbenicillin	Ciprofloxacin	Cefoxitin	Fusidic Acid	Imipenem	Levofloxacin	Moxifloxacin	Rifampicin	Tetracycline	Vancomycin	Metronidazole
Acinetobacter Baumannii	-	-	-	-	-	0.125	-	-	-	-	-	-
Bacillus Subtilis	-	0.25	0.125	-	-	-	-	-	-	0.5	-	-
Campylobacter Jejuni	0.5	-	0.25	-	-	-	0.25	0.125	-	-	-	-
Escherichia coli 0157 H:7	>16	-	0.125	-	-	-	-	-	4	1	>16	-
Listeria Monocytogenes	0.25	8	-	-	-	-	-	-	-	0.5	0.5	-
Klebsiella Pneumoniae	-	-	0.125	0.25	-	0.25	-	-	-	-	-	-
Pseudomonas Aeruginosa	-	0.25	0.125		-	0.125	-	-	-	-	-	-
Salmonella Typhimurium	-	-	0.125		-	1	-	-	-	-	-	-
Streptococcus Pneumoniae	-	-	0.125		-	-	0.125	0.125	-	-	-	-
Staphylococcus Aureus	0.5	0.5	-		0.125	-	-	-	0.125	-	-	-

* Each value represents the mean of three observations.

Discussion

The inclusion of commonly used antibiotics served three purposes. The first is as a quality control measure, by aiding in the determination that strains are susceptible to antibiotics that they should be. Second of all, we were able to determine if the specific strains being tested were resistant to commonly-used antimicrobials. Third, they were useful for comparison of the activity of our compounds.

The *A. baumannii* strain used was susceptible to the antibiotic imipenem based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [84]. *A. baumannii* has been proven to be multi-drug resistant and to have unpredictable susceptibility patterns [85]. Therefore, replacements to conventional therapies are always needed. Compounds 1 and 3 show a high level of inhibitory activity on this bacterial species and could be considered for further study.

B. subtilis was considered susceptible to the antibiotic ciprofloxacin based on EUCAST breakpoints. All four compounds inhibited this species of bacteria with an MIC of 1 µg/ml or lower, and compounds 1, 3, and 4 all inhibited at a level equivalent to the commonly-used antibiotic tetracycline (MIC = 0.5 µg/ml)

Based on clinical breakpoints, the C. jejuni species used was susceptible to the antibiotics erythromycin and ciprofloxacin. While all four compounds displayed inhibitory activity of equal to or less than 2 µg/ml, compound 4 was the most effective, inhibiting at 0.25 µg/ml, equivalent to the commonly used antibiotics ciprofloxacin and levofloxacin.

The strain of E. coli 0157H7 tested was susceptible to the commonly-used antibiotic ciprofloxacin, but was not inhibited by erythromycin or vancomycin even at 16 µg/ml. Of the four compounds tested, only compound 3 was able to inhibit the growth of the bacteria.

Compound 3 inhibited the strain of E. coli at 4 µg/ml, but it is also important to note that the original *M. unguiculata* extract displayed equivalent activity. Both the compound mixture and the extract displayed activity at the level of the antibiotic rifampicin.

The *L. monocytogenes* strain tested displayed susceptibility to the antibiotic erythromycin based on EUCAST breakpoints. Compound 1 was found to have the strongest inhibitory activity of the isolated compounds at MIC of 0.5 µg/ml. This is equivalent to two commonly used antibiotics, tetracycline and vancomycin. Compounds 3 and 4 displayed inhibitory activity at 2 µg/ml, proving they too have potential applicability as replacement antimicrobials for this common pathogen.

K. pneumoniae was susceptible to the commonly used antibiotics ciprofloxacin and imipenem in this test. Compound 3 was the only isolated product to inhibit this species of bacteria. The MIC of 4 µg/ml was equivalent to the MIC of the *M. unguiculata* extract, which leads to the belief that other active compounds exist within the extract and together create an additive effect.

P. aeruginosa was susceptible to the commonly used antibiotics ciprofloxacin and imipenem. Compound 4 showed the most prominent activity against *P. aeruginosa*, with an MIC of 0.25 µg/ml; equivalent to the antibiotic carbenicillin. The MIC of compound 1 was 0.5 µg/ml, proving either compound could be a potential alternative for current pharmaceuticals.

S. typhimurium was susceptible to the two antibiotics commonly used for its treatment; ciprofloxacin and imipenem. Only compound 3 displayed inhibitory activity on this species of bacteria (MIC = 4 µg/ml). Furthermore, the MIC of the isolated mixture of compounds was higher than the MIC for the *M. unguiculata* extract.

S. pneumoniae was susceptible to ciprofloxacin, levofloxacin, and moxifloacin. Compounds 1 and 4 displayed a high level of activity with MICs of 0.5 µg/ml.

S. aureus was proven to be susceptible to antibiotics erythromycin, fusidic acid, and rifampicing based on EUCAST breakpoints. All of the isolated products tested inhibited this strain of bacteria at or below 1 µg/ml. Compound 4 displayed the greated activity, with an MIC of only 0.125 µg/ml, which is equivalent to or greater than all the antibiotics included.

In conclusion, the four compounds isolated displayed broad-spectrum antimicrobial activity. All of the compounds were successful at inhibited both gram-positive and gram-negative bacteria. The gram-negative bacteria posed a greater challenge to the compounds, as compounds 1, 2, and 4 failed to inhibit the same three gram negative bacteria; *E. coli* 0157: H7, *K. pneumoniae,* and *S. typhimurium*. These three bacterial species are all part of the same family, known as enterobaceriaceae. Furthermore, compounds 2 and 4 also failed to inhibit gram negative *A. baumannii* at concentrations up to 16 µg/ml. The isolated mixture of compound 3 successfully inhibited the gram-positive bacteria as well as these more challenging gram negative bacteria, proving superior antimicrobial ability. Unfortunately, for the most part, fractionation of *M. unguiculata* did not lead to improved activity over the original extract. Further testing would likely lead to other active compounds in the *M. unguiculata* extract. Combining a number of isolated compounds would likely produce a stronger effect than the original extract, however, the low MIC values obtained prove that *M. unguiculata* extract may have potential antimicrobial use even without fractionation.

Minimum Bactericidal Concentration (MBC)

Introduction

The MIC test proves the inhibitory activity of the sample tested; however, it does not indicate the action of the antibacterial agent. Researchers often improve the MIC testing by testing to see if growth of the microorganism will begin again following the removal of the antibacterial agent. Therefore, it is important to follow-up this test by determining the minimal bactericidal concentration (MBC) of the sample. MBC is the lowest concentration of an antibiotic to kill 99.9% of a bacterial inoculum [82]. Researchers use these two tests to determine the mode of action of the antimicrobial compound, being either bacteriostatic or bactericidal.

Materials and Methods

Following the broth microdilution test for MIC, 20 µl of suspension was removed from each microtitre plate well showing no growth, and transferred to a new well containing 240 µl of sterilized broth. Following a 24 hour incubation period at 37 ºC in appropriate conditions, the results were obtained by measuring absorbance at 625 nm. The MBC was determined as the lowest concentration of antibacterial agent in the original MIC test sample to cause a one-thousand fold reduction in bacterial growth compared with the growth control (99.9% killing).

Results

The effect of compound 1 on the bacterial species *A. baumannii* is considered bactericidal as the MIC and MBC are equal. For the remaining bacterial species inhibited by compound 1, a 1 to four-fold increase in concentration was required to obtain a bactericidal effect; with the exception of *B. subtilis* with an MBC 32-fold greater than the recorded MIC (Table 11).

Similarly, a bactericidal effect was seen at the MIC of compound 2 on the bacterial species *C. jejuni* and *L. monocytogenes*. By increasing the concentration by 1 to 4fold more than the MIC value, a bactericidal effect could be produced for the other bacterial species (Table 12). Against *B. subtilis*, the bacteriocidal effect was not evident even up to concentrations of 16 μg/ml. In most cases, the original *P. papyracea* extract exhibited stronger bactericidal effect than the isolated compound 2.

The mixture of compounds present in compound 3 displayed strong bactericidal activity with MBCs equal to the MICs for *A. baumannii*, *C. jejuni*, *E. coli* 0157:H7, *K. pneumoniae*, *P. aeruginosa*, and *S. pneumoniae* (Table 13). MBCs were slightly increased from the MICs for *L. monocytogenes*, *S. typhimurium*, and *S. aureus*. Finally, 16 μg/ml of the compound mixture was required to obtain a bactericidal effect on the bacterial species *B. subtilis*, although the MIC was 0.5 μg/ml.

Finally, compound 4 exerted bactericidal activity on *S. pneumoniae* at the MIC of 0.5 μg/ml. A bactericidal effect could be seen at 2-8x the MIC for all other bacterial species involved (Table 14).

Table 11 *The minimum bactericidal concentration (MBC) of bacterial growth of bacterial strains exerted by Mammea africana plant crude extract and the isolated coumarin mammea A/AA; compound 1.*

	MBC (µg/ml)*	
	Crude *Mammea Africana* Extract	Compound 1
Acinetobacter Baumannii	>16	2
Bacillus Subtilis	16	16
Campylobacter Jejuni	4	2
Escherichia coli 0157 H:7	>16	>16
Klebsiella Pneumoniae	>16	>16
Listeria Monocytogenes	2	1
Pseudomonas Aeruginosa	2	1
Salmonella Typhimurium	>16	>16
Staphylococcus Aureus	4	2
Streptococcus Pneumoniae	0.25	0.5

* Each value represents the mean of three observations.

Table 12 *The minimum bactericidal concentration (MBC) of bacterial growth of bacterial strains exerted by Phyllospongia papyracea plant crude extract and the isolated tetrabrominated diphenyl ether; compound 2.*

	MBC (µg/ml)*	
	Crude *Phyllospongia papyracea* Extract	Compound 2
Acinetobacter Baumannii	>16	>16
Bacillus Subtilis	4	>16
Campylobacter Jejuni	2	2
Escherichia coli 0157 H:7	>16	>16
Klebsiella Pneumoniae	>16	>16
Listeria Monocytogenes	1	8
Pseudomonas Aeruginosa	1	8
Salmonella Typhimurium	>16	>16
Staphlococcus Aureus	1	2
Streptococcus Pneumoniae	2	4

* Each value represents the mean of three observations.

Table 13 *The minimum bactericidal concentration (MBC) of bacterial growth of bacterial strains exerted by Monanchora unguiculata plant crude extract and the isolated active mixture of compounds; compound 3.*

	MBC (µg/ml)*	
	Crude *Monanchora unguiculata* Extract	Compound 3
Acinetobacter Baumannii	4	4
Bacillus Subtilis	>16	16
Campylobacter Jejuni	2	2
Escherichia coli 0157 H:7	4	4
Klebsiella Pneumoniae	16	4
Listeria Monocytogenes	4	4
Pseudomonas Aeruginosa	4	2
Salmonella Typhimurium	4	8
Staphylococcus Aureus	2	4
Streptococcus Pneumoniae	2	2

* Each value represents the mean of three observations.

Table 14 *The minimum bactericidal concentration (MBC) of bacterial growth of bacterial strains exerted by Dysidea herbacea plant crude extract and the isolated active compound; compound 4.*

	MBC (µg/ml)*	
	Crude *Dysidea herbacea* Extract	Compound 4
Acinetobacter Baumannii	>16	>16
Bacillus Subtilis	8	4
Campylobacter Jejuni	2	1
Escherichia coli 0157 H:7	>16	>16
Klebsiella Pneumoniae	>16	>16
Listeria Monocytogenes	2	4
Pseudomonas Aeruginosa	0.5	1
Salmonella Typhimurium	>16	>16
Staphylococcus Aureus	0.5	2
Streptococcus Pneumoniae	0.5	0.5

* Each value represents the mean of three observations.

Table 15 *The minimum bactericidal concentration (MBC) on bacterial growth of bacterial strains exerted by commonly used antibiotics.*

	Antibiotic MBC (µg/ml)*											
	Erythromycin	Carbenicillin	Cefoxitin	Ciprofloxacin	Imipenem	Fusidic Acid	Levofloxacin	Moxifloxacin	Rifampicin	Tetracycline	Vancomycin	Metronidazole
Acinetobacter Baumannii	-	-	-	-	0.25	-	-	-	-	-	-	-
Bacillus Subtilis	-	4	-	0.5	-	-	-	-	-	4	-	-
Campylobacter Jejuni	0.5	-	-	0.5	-	-	0.25	0.125	-	-	-	-
Escherichia coli 0157 H:7	>16	-	-	0.125	-	-	-	-	8	8	>16	-
Klebsiella Pneumoniae	-	-	16	0.125	0.25	-	-	-	-	-	-	-
Listeria Monocytogenes	0.5	16	-	-	-	-	-	-	-	2	1	-
Pseudomonas Aeruginosa	-	1	-	1	0.25	-	-	-	-	-	-	-
Salmonella Typhimurium	-	-	-	0.125	4	-	-	-	-	-	-	-
Staphyloccus Aureus	0.5	2	-	-	-	0.5	-	-	0.125	-	0.5	-
Streptococcus Pneumoniae	-	-	-	0.125	-	-	0.125	0.125	-	-	-	-

* Each value represents the mean of three observations.

Discussion

In most cases, the MIC represents a bacteriostatic activity. By increasing the concentration above the MIC, a bactericidal activity can be produced. Although breakpoint values only exist for MIC values, this trend is also true of the commonly used antibiotic agents, of which most required an increased concentration for their activity to be considered bactericidal.

Compounds 1, 2, and 3 could only produce a bactericidal effect on *B. subtilis* bacteria at high concentrations (MBC 16 μg/ml or above). Compound 4 displayed a far superior bactericidal activity on this bacterial species, with a low MBC of 4 μg/ml, making a good candidate for further drug development.

The mixture of compounds present in compound 3 displayed significant bactericidal activity against all the bacterial species included except *B. subtilis*. It can be concluded that this mixture of compounds has strong broad-spectrum bactericidal activity, which deserves further investigation.

Similar to the results of MIC testing, MBC testing showed decreased MBC values when the isolated product was compared with the original extract, only in the cases of compounds 1 and 4. Compounds 2 and 3 often showed a higher MBC value than the original extract which further solidifies the hypothesis that some bioactive compounds were lost during fractionation. A second possible explanation is that the isolated compounds themselves degrade over time, losing activity, while the original extract is significantly more stable, and does not lose activity during storage.

CHAPTER 4: APPLICABILITY OF ISOLATED COMPOUNDS

Introduction

Stability testing evaluates the effect of environmental factors on the efficacy of a drug substance [86]. Stability testing to ensure product quality, safety, and efficacy are required prior to the approval of any pharmaceutical product. At the developmental stage, stability studies provide information required to determine shelf-life, packaging, and storage conditions [86]. The influence of environmental factors on the activity of the product being developed must be determined as temperature, humidity, and light are known to have a detrimental effect on some active compounds. In packaged foods, growth and survival of common spoilage and pathogenic microorganisms such as *L. monocytogenes, E. coli* 0157:H7, *S. enterica* serovar typhimurium, *S. aureus, and C. Jejuni*, are affected by a variety of intrinsic factors, such as pH and oxygen presence, as well as extrinsic factors of storage conditions, including temperature, time, and relative humidity [34]. The temperature of cooking may affect the antimicrobial compounds of natural products in foodborne pathogens. Therefore, heat treatment is required to determine if the antimicrobial compound has potential use as a commercial antimicrobial which could be applied to food products [87]. Stress testing of compounds often requires treatment with high heat, which serves not only the purpose of addressing the compound's ability to withstand temperature associated with food storage, processing, cooking, or sterilization, but also as a method of accelerated aging. In this way, a compound that can withstand heat treatment at a high temperature is considered more likely to remain stable over long periods of storage.

Foods have various pH levels, usually ranging from pH 3 for foods such as cheese and pasta up to pH 10 for foods such as spinach and broccoli. If an antimicrobial agent becomes ineffective at a specific pH level, this will limit its potential application as a food preservative.

The role of pH level is important to consider as it has an effect on the growth rate of microbes, as well as the interaction of antimicrobial agent and microbial growth; affecting the MIC of the agent. The optimal pH for bacterial growth is usually between ph 6-8, however, even foods with pH levels at the far ends of the spectrum are subject to spoilage and/or pathogenic bacteria. Antimicrobial agents also have a specific pH at which they display optimal activity. Food preservatives have known optimal pH levels which are usually reflects the active moiety of the molecule and determines the application that the specific agent may be used for.

Finally, the shelf-stability of a potential pharmaceutical agent must be considered during the development stage of any drug. The optimal conditions of storage must be determined to facilitate proper packaging and storage instructions, as well as to estimate the product's shelf-life. Most products will remain stable longer if stored at lower temperatures, however, it is most convenient to store pharmaceutical products at room temperature. Exposure of the product to light is also important to consider as light is known to cause compound degradation, which will significantly impact the activity of a natural antimicrobial. Intermediate length testing should cover a minimum of six months duration, however, it is considered unnecessary to continue testing if a significant change in efficacy is seen in the first three months [88]. In general, a loss of activity up to 85% can lead to failure in therapy, and is considered significant loss of activity [86].

Materials and Methods

Thermal Stress Testing - Isolated compounds (100 µg/ml in water) were heated to 37ºC, 50ºC, 80ºC, 100ºC, and 121ºC for 15 minutes using a Fisher Scientific Isotemp hot plate. Samples were removed to ice for 10 minutes for immediate cooling. Following heat treatment, the level of bioactivity of the compounds was assessed via broth microdilution method as

previously described [89]. Bacterial colonies of *B. subtilis* were selected from 24 h cultures, suspended in sterile distilled water and adjusted to match a 0.5 McFarland standard by measuring absorbance at 625 nm using an HTS 7000 Bio Assay Reader (Perkin Elmer, Norwalk, CT). The bacterial cell numbers were adjusted to approximately 1 x 105 colony forming units (CFU)/ml. Isolated active compounds stored in the freezer and compounds which had undergone heat treatment were subjected to serial two-fold dilution in a 96-well microtiter plate with final test concentrations ranging from .0125 to 16 µg/ml. Assay plates were incubated at 37 ºC for 24 h, and growth was assessed by measuring absorbance at 625 nm using the Bio Assay Reader. MIC's of antibiotic agents was interpreted as the lowest concentration of antibiotic agent to cause an 80% or greater reduction of growth compared to the growth control. The growth control consisted of DMSO diluted in sterile distilled water (1:9), and broth, but no test samples. A blank containing no bacteria was also included. The assays were performed three times and MIC values were reported as the mean value of each assay.

Effect of pH Treatment - Buffers were prepared at pH 3, 4, 5, 6, 8, 9, and 10. Stock solutions of active compounds (1 mg/ml in DMSO) were evaporated under the fume hood overnight. Buffers or sterile distilled water (pH 7) were added to dried samples to achieve samples with a final concentration of 100 µg/ml. Samples were mixed using a vortex mixer. The level of bioactivity of the compounds was assessed via broth microdilution method as previously described. Bacterial colonies of *B. subtilis* were selected from 24 h cultures, suspended in sterile distilled water and adjusted to match a 0.5 McFarland standard by measuring absorbance at 625 nm using an HTS 7000 Bio Assay Reader (Perkin Elmer, Norwalk, CT). The bacterial cell numbers were adjusted to approximately 1 x 10^5 colony forming units (CFU)/ml. Isolated active compounds treated with buffers at various pH levels were subjected to serial two-fold dilution in

a 96-well microtiter plate with final test concentrations ranging from .0125 to 16 µg/ml. Assay plates were incubated at 37 ºC for 24 h, and growth was assessed by measuring absorbance at 625 nm using the Bio Assay Reader. MIC's of antibiotic agents was interpreted as the lowest concentration of antibiotic agent to cause an 80% or greater reduction of growth compared to the growth control. A set of growth controls was included in this assay in order to serve as a control for variations in growth of *B. subtilis* bacteria based on the inclusion of the buffers in the concentration range of .0125 to 16 µg/ml in the growth medium [90]. A blank containing no bacteria was also included.

Storage Conditions - Samples of isolated active compounds 1-4 (100 µg/ml in sterile distilled water) were placed in various storage conditions. The conditions chosen for testing were: -20ºC (freezer), 4ºC (fridge), room temperature without light exposure, and room temperature with light exposure. Samples were removed from storage at one month intervals and bioactivity was assessed by broth microdilution as previously described. Bacterial colonies of B. subtilis were selected from 24 h cultures, suspended in sterile distilled water and adjusted to match a 0.5 McFarland standard by measuring absorbance at 625 nm using an HTS 7000 Bio Assay Reader (Perkin Elmer, Norwalk, CT). The bacterial cell numbers were adjusted to approximately 1 x 10^5 colony forming units (CFU)/ml. Isolated active compounds treated with varying storage environments were subjected to serial two-fold dilution in a 96-well microtiter plate with final test concentrations ranging from .0125 to 16 µg/ml. Assay plates were incubated at 37 ºC for 24 h, and growth was assessed by measuring absorbance at 625 nm using the Bio Assay Reader. MIC's of antibiotic agents was interpreted as the lowest concentration of antibiotic agent to cause an 80% or greater reduction of growth compared to the growth control. The growth control consisted of DMSO diluted in sterile distilled water (1:9), and broth, but no

test samples. A blank containing no bacteria was also included. They were tested for a period of 6 months.

Results

Thermal Stress Testing - Compound 1 did not show any loss in activity following heat treatment at 50 ˚C. Heat treatment at 80 ˚C caused a slight loss in activity which became more pronounced with treatment above 100 ˚C. Although this compound proved to be susceptible to heat treatment, even following treatment at 121 ˚C for 15 minutes, the compound was still able to inhibit the B. subtilis bacteria at a low MIC of 2 µg/ml (Figure 10).

Thermal stability testing proved that compound 2 was the most susceptible to heat treatment. It had significant loss of activity against *B. subtilis* even when heated only to body temperature 37 ˚C. Following heat treatment of 80 ˚C for 15 minutes, the compound did not inhibit the growth of *B. subtilis* bacteria even at the highest tested concentration of 16 µg/ml.

The mixture of compounds present in compound 3 was determined to be highly heat-stable. Following heat treatment at 121 ˚C, the antimicrobial activity was only slightly decreased, producing a low MIC of 1 µg/ml.

Compound 4 maintained its activity following heat treatment of 100 ˚C (MIC = 0.5 µg/ml). It showed decreased activity following heat treatment at 121 ˚C displaying an MIC which increased to 4 µg/ml.

Effect of pH Treatment - None of the pH buffers on their own significantly inhibited the growth of the *B. Subtilis* bacteria, although, as expected, the highest level of bacterial growth was recorded when buffers at pH of 6 and 7 were included. This proves that even small amounts of basic or alkaline buffer will decrease the growth rate of the *B. subtilis* bacteria. Compound 1 did not show any inhibition on the *B. Subtilis* even up to a concentration of 16 µg/ml. Compound

2 showed inhibition at the MIC level of 1 µg/ml with pH buffer 10 only. With pH buffer 9, the bacteria was inhibited at 4 µg/ml. Compound 3 showed inhibition at 16 ppm when buffers at pH 3, 5, and 7 were included. Finally, compound 4 showed inhibition at 1 µg/ml when buffers at ph 9 and 10 were included and at 4 µg/ml with buffer at pH 8.

Storage Conditions - Compound 2 showed significant loss of activity following storage at -20 ˚C by 2 months time. By 6 months, compound 2 displayed an MIC of 16 µg/ml, compared to an MIC of 1 µg/ml at Day 1. Compound 1 retained its bioactivity throughout 4 months of storage at -20 ˚C, however, at month 5 the compound displayed a higher MIC of 8 µg/ml. Compounds 3 and 4 exhibited strong antimicrobial activity against B. subtils following 6 months of storage at -20 ˚C. At the 6 month time-point both compounds exhibited an MIC of only 2 µg/ml (Figure 11).

Fridge storage at 4 ˚C produced similar results to those obtained following storage in the freezer (Figure 12). Compound 2 showed significant instability and at month 6 did not inhibit *B. subtilis* even at the highest tested concentration of 16 µg/ml. Compound 1 remained stable until 4 months, but showed a significant reduction in activity at month 5 and 6. Compounds 3 and 4 remained relatively stable throughout the test period.

Compound 2 appeared to be the least stable of the compounds when stored at room temperature without light exposure. It exhibited an MIC of 8 µg/ml against *B. subtilis* at month 2 and 3, which increased to 16 µg/ml by month 4. At 6 months time the compound no longer displayed bioactivity against the bacteria even at the highest tested concentration of 16 µg/ml. Compound 1 was the second least stable of the compounds stored in this environment. At month 5, compound 1 displayed an MIC of 8 µg/ml against *B. subtilis*, which rose to 16 µg/ml at month 6. Compound 3 was able to retain its bioactivity following 6 months of storage at room

temperature without exposure to light. Following the 6 month storage period, the mixture of compounds exhibited the low MIC of 2 μg/ml (Figure 13). Finally, compound 4 showed it is susceptible to higher storage temperatures as it displayed an increased MIC of 4 μg/ml at month 5 and 6.

As displayed in Figure 14, the activity of the isolated compounds was decreased following storage at room temperature with light exposure. Compound 2 displayed an MIC of 16 μg/ml against *B. subtilis* following only 4 months of storage. Compound 1 had lost its inhibitory activity against *B. subtilis* at all tested concentrations by 6 months time. Compound 3 showed the most stability, exhibiting an MIC of 4 μg/ml following the test period. Finally, compound 4 displayed an MIC of 8 μg/ml following 6 months of storage at room temperature with exposure to light.

Figure 10 *Minimum inhibitory concentration (MIC) of heat treated compounds against B. subtilis bacteria.*

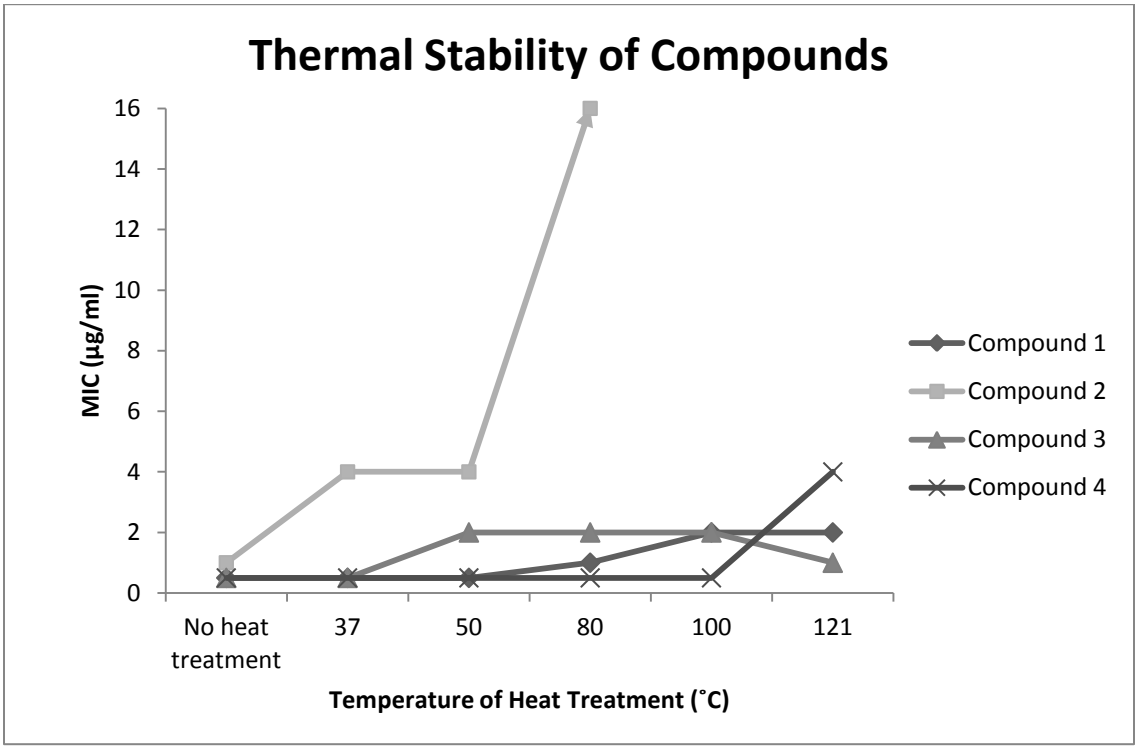


Figure 11 *Minimum inhibitory concentration (MIC) of compounds stored at -20 ˚C against B. subtilis bacteria.*

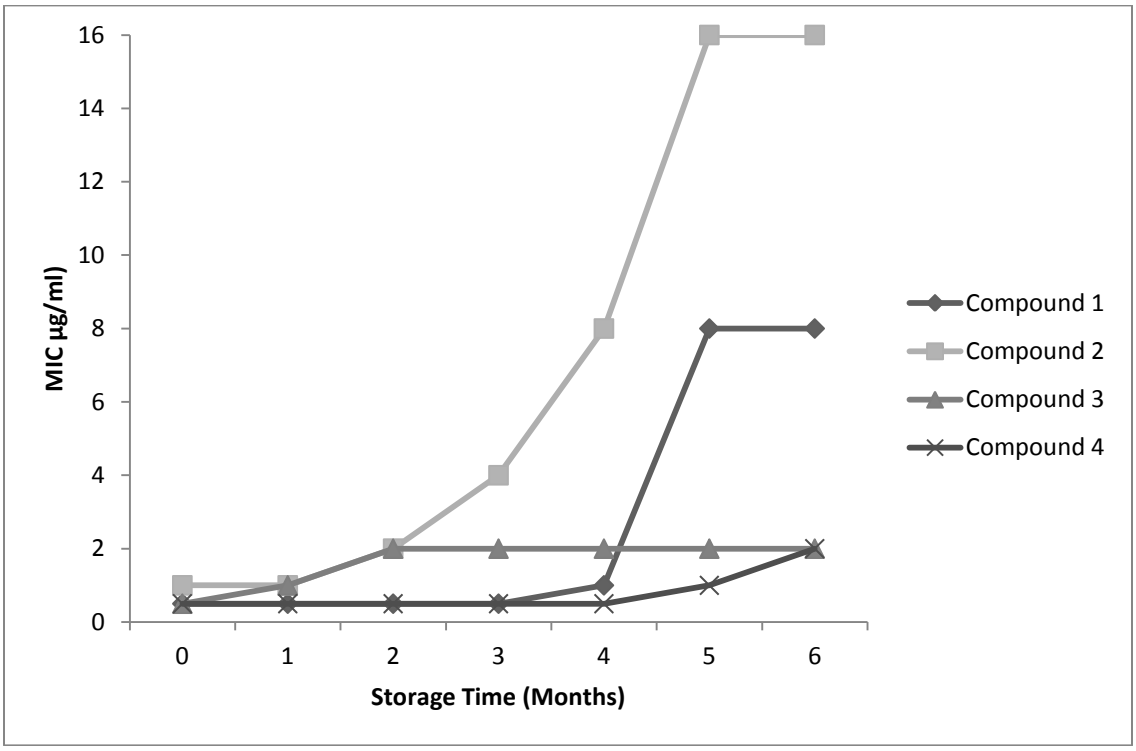


Figure 12 *Minimum inhibitory concentration (MIC) of compounds stored at 4 ˚C against B. subtilis bacteria.*

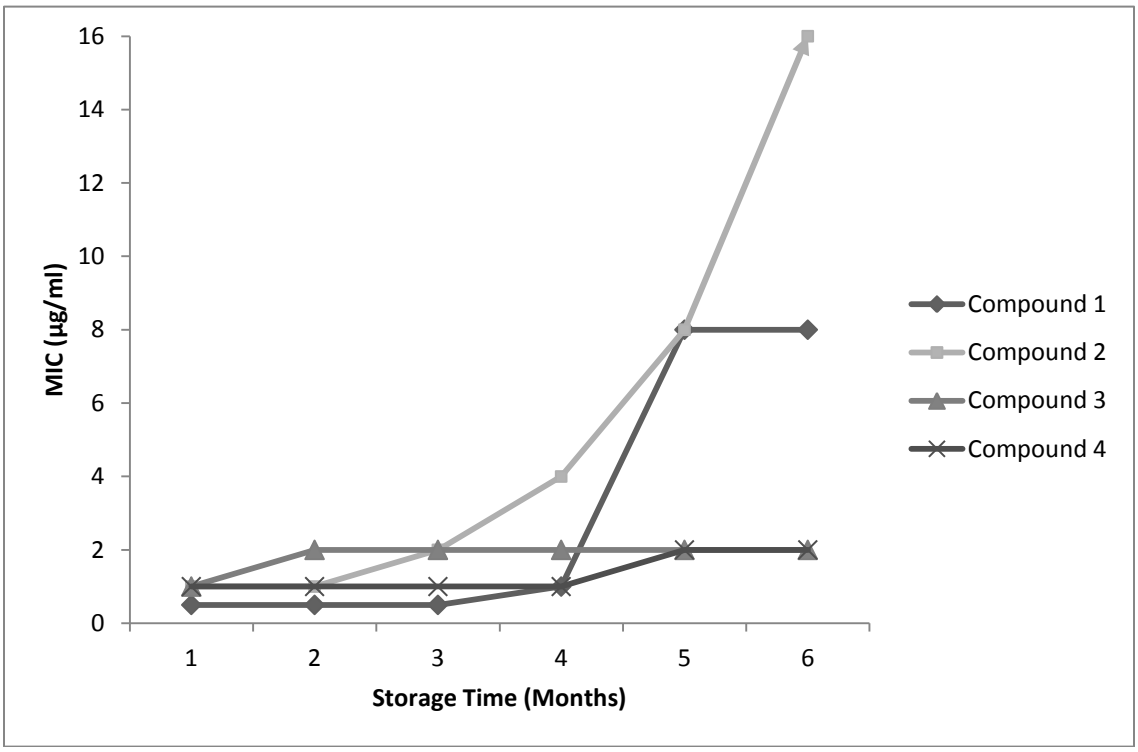


Figure 13 *Minimum inhibitory concentration (MIC) of compounds stored at room temperature without light exposure against B. subtilis bacteria.*

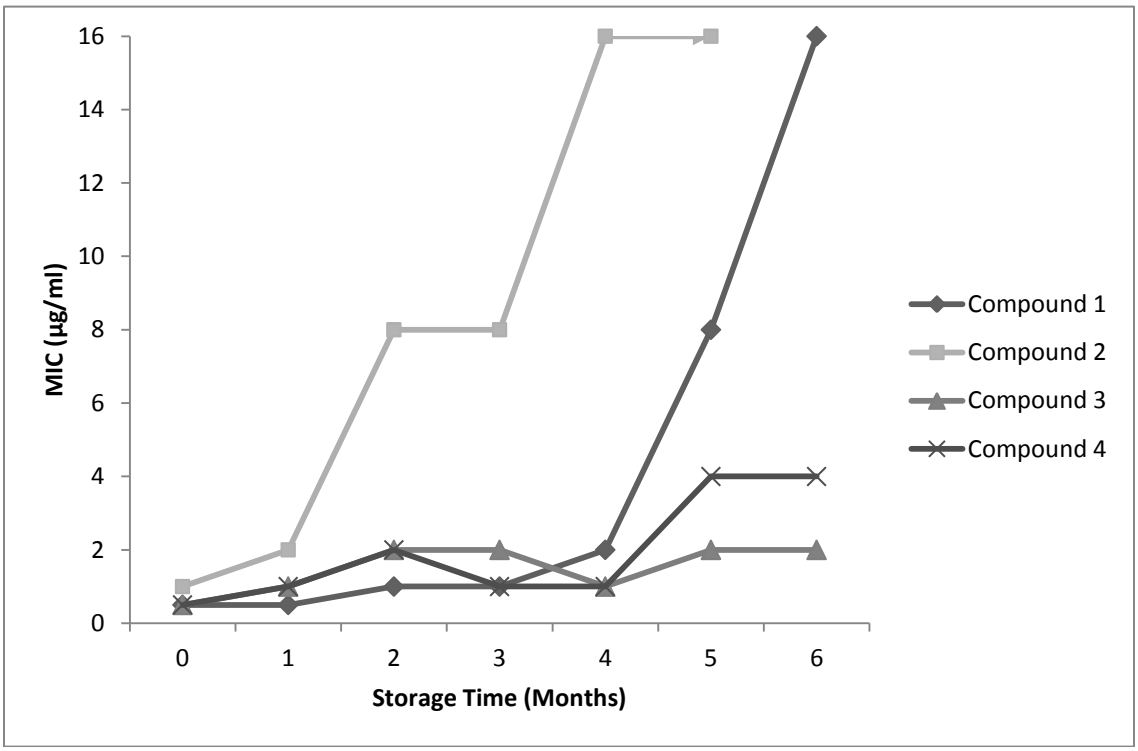
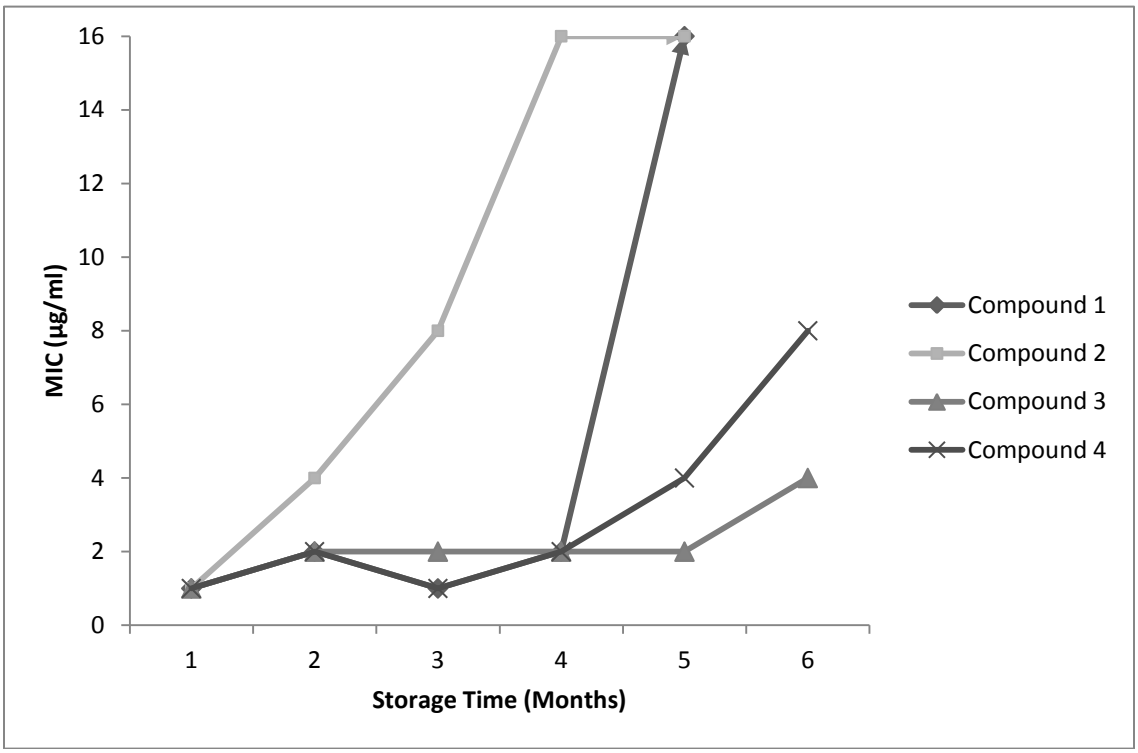


Figure 14 *Minimum inhibitory concentration (MIC) of compounds stored at room temperature with light exposure against B. subtilis bacteria.*



Discussion

Thermal Stress Testing - Compounds isolated from plant and marine organisms were subjected to various levels of heat treatment. Only compound 2 showed a significantly decreased activity level at human body temperature of 37 ˚C. This compound may not be stable enough to be used as a pharmaceutical agent, as it may not provide the desired effect in such circumstances. Furthermore, compound 2 was not able to inhibit the *B. subtilis* bacteria following heat treatment at 80 ˚C even at the highest tested concentration of 16 μg/ml. Thermal treatment of antimicrobial agents which resulted in a reduction of antimicrobial activity suggests compound degradation [91]. The potential applications of compound 2 are limited as the results of the thermal stress test lead to the fact that compound 2 is unlikely to hold up to long-term stability tested even at lower temperatures.

The remaining compounds maintained a high level of activity following heat treatment at temperatures up to 121 ˚C. This proves that these compounds may have potential application as pharmaceutical agents, or food preservatives. The concentration used may need to be increased to compensate for the 8-fold reduction of activity of compound 4 at 121 ˚C. The results also lead to the hypothesis that compounds 2 and 3 are the most likely to remain active following more long-term shelf stability testing.

Effect of pH Treatment - The compounds were originally dissolved in DMSO to produce the stock solution which was stored in the freezer. Stock solutions were dried under the fume hood overnight, so that the compounds were able to be rehydrated in buffer and access the effect of the varying pH level on the efficacy of the antimicrobial agent. Unfortunately, dissolving the compounds directly in buffer was not possible. The results prove that the compounds had significant loss of activity following the preparation of the samples. This is apparent by the fact

that none of the compounds performed as expected even when only water was added to the CAMHB broth. There are two possible explanations for the results that were obtained. The first explanation is that the active compounds were volatile and lost activity during the drying of the stock solution. A second possibility is that the active compounds bound to the vial when the DMSO was evaporated, and when buffer was added, the active compound did not become incorporated into the solution, even with vortex mixing. Compounds 2 and 4 did show inhibitory activity at the low concentration of 1 μg/ml at a few specific pH levels, however, there is not sufficient data to support any findings on the effect of pH on the isolated active compounds. It is most probable that buffers of pH 9 and 10 were the most successful at dissolving compounds 2 and 4. Although DMSO and distilled water have similar pH levels, DMSO is an aprotic solvent while water represents a protic solvent and has a higher polarity. These differences are likely responsible for the unexpected results.

Storage Conditions - As expected, all the compounds were found to be susceptible to variations in storage conditions. Compound 1 displayed an increased stability when stored at fridge or freezer temperature. Furthermore, exposure to light had an effect on the bioactivity of the compound. The applicability of this compound is limited as it is not shelf-stable for an extended period of time.

Compound 2 was the least stable of the compounds examined. In all storage conditions the compound displayed significant loss of bioactivity against *B. subtilis*. Only when stored at -20 ˚C did the compound show any inhibitory activity at month 6 (MIC of 16 μg/ml). This compound has shown instability in all tests, and would not be chosen for further development for this reason.

The mixture of compounds present in compound 3 retained activity following 6 months of storage in all of the tested environments. This compound is the most likely to be shelf-stable for an extended period of time.

Compound 4 was the second most stable of all the compounds tested. It retained inhibitory activity in all tested environments. This compound was susceptible to light, so opaque packaging would be recommended to increase the shelf-life of this product.

CHAPTER 5: CYTOTOXICITY OF ISOLATED COMPOUNDS

Introduction

Plants have been eaten and used in traditional medicine for centuries. This leads many to believe that all compounds derived from natural sources are safe for human consumption. However, an isolated active compound requires testing to ensure it is safe in its purified form, and at the level required to inhibit the bacterial growth. Plant research is currently separated into ethnopharmacology (ex. medicinal herbs) and toxicology (ex. poisonous plants). Both types of research have led to the production of drugs and lead compounds [34]. Compounds are often tested using an *in vitro* cell culture system and cytotoxicity is determined by the effect of the compound on the cell growth rate [92]. MTS assay represents a well established measurement system for cytotoxicity. The assay relies on the conversion of a tetrazolium salt into a coloured formazan product by mitochondrial activity of viable cells. The amount of living cells in the culture is directly proportional to the amount of formazan produced and is measured at 492 nm [93]. The assay is known to produce precise results in a timely manner [94].

Materials and Methods

To fulfill this aim, the cytotoxicity of isolated active compounds was determined by MTS assay using mouse embryonic fibroblast (MEF) cell line, NIH 3T3 obtained from ATCC. MTS assay was conducted as previously described [95]. Active compounds were dissolved in DMSO to prepare a stock solution with a concentration of 1 mg/ml. The stock solution was diluted in sterile distilled water to obtain samples with a concentration of 250 µg/ml. The NIH 3T3 cells (5×10^3) were seeded in a 96-well culture plate and after overnight incubation the medium was removed and replaced with a fresh medium containing isolated active compounds at 50 µ/ml, 25 µg/ml. Controls were included that did not contain any test substance (1:3 water:

DMSO or 1:7 water: DMSO). After 72 h of incubation, 20 µl of CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI) was added to each well. After 2 h incubation at 37 °C in a humidified, 5 % CO_2 atmosphere, the absorbance at 485 nm was recorded on an ELx800 plate reader (Bio-Tek, Winooski, VT). The untreated control of every experiment was defined as 100% proliferation and the values of the antimicrobial-treated cultures were calculated in relation to the control. Each variant of the experiment was performed in octuplet.

Results

Cell proliferation studies of the isolated active compounds showed varying degrees of cytotoxicity using the NIH 3T3 MEF cell line. At a concentration of 25 µg/ml, all compounds exhibited significant cytotoxicity (Table 16). Compounds 1 and 4 displayed 40.5 and 41.6% proliferation, respectively. The MEF cell line was further diminished by treatment with compounds 2 and 3 at 25 µg/ml, resulting in only 9.8% and 5.4% cell proliferation, respectively.

When the concentration of isolated active compounds was increased to 50 µg/ml, the results were more pronounced. The MEF cell line treated with compounds at 50 µg/ml resulted in almost complete obliteration of cell viability. Cell proliferation was between 0.32% and 3.9% following treatment with isolated active compounds 1-4 at a concentration of 50 µg/ml (Table 17).

Figure 15 *MTS assay of NIH 3T3 cell proliferation caused by isolated active compounds at a concentration of 25 µg/ml.*

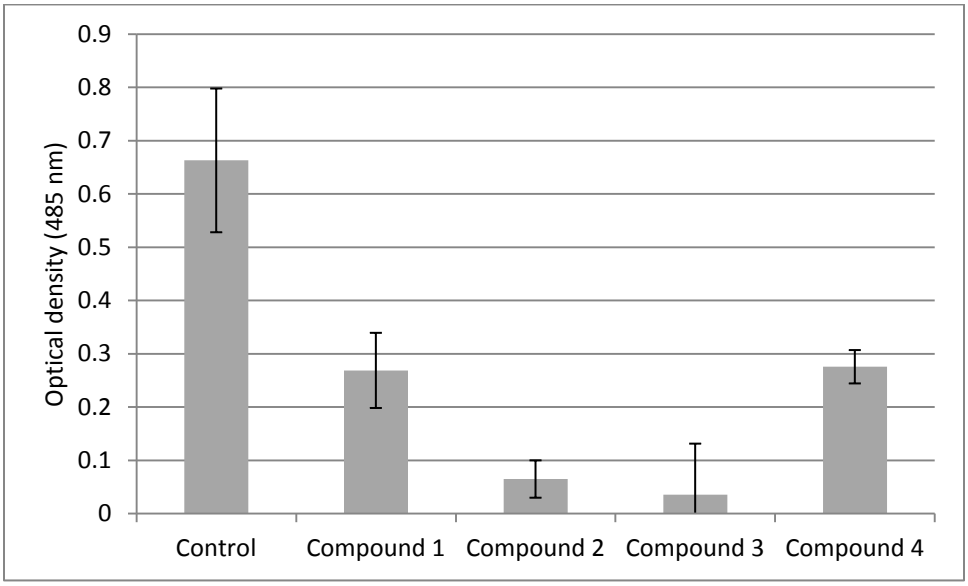
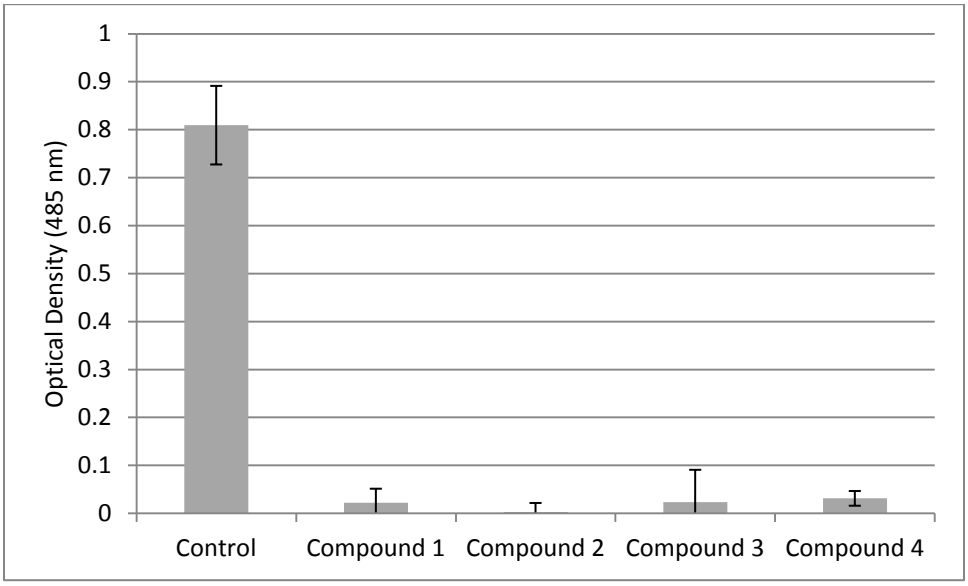


Table 16 *MTS assay of NIH 3T3 cell proliferation caused by isolated active compounds at a concentration of 50 µg/ml.*



Discussion

All isolated compounds were found to be cytotoxic to the mouse embryonic fibroblast (MEF) cell line, NIH 3T3. Although this has important implications in the applicability of the compounds and their further development, it is not uncommon for the current pharmaceutical antibiotics to be cytotoxic to normal human cells. Researchers tested the cytotoxicity of common antibiotics ciprofloxacin, moxifloxacin, erythromycin against primary human osteoblasts. The commonly used antibiotics were found to display cytotoxicity, however higher concentrations were tested (400 μg/ml) [96]. Researchers often determine the specific mechanism by which an agent exerts cytotoxicity. After isolating the cause of the cytotoxicity, it is possible to modify the structure of the compound to remove its cytotoxic effect, while keeping its desirable antimicrobial activity. This process of molecular modification is a step in the drug development process which is often carried out when a lead compound with exceptional bioactivity is discovered to exhibit cytotoxicity [97].

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

The ongoing development of resistance of pathogenic bacteria to antibiotics represents a major threat to public health. The issue is exacerbated by the fact that new drugs rarely succeed in being fully developed and made available to consumers [98]. Natural products have seemingly endless chemical diversity and are central to current pharmaceutical development [98]. The primary aim of this project was to identify and characterize components of plant and marine extracts with inhibitory activity against those bacterial species which are considered high priority due to antimicrobial resistance and high incidence of occurrence.

In this study, extracts of plant and marine organisms were screened for antimicrobial activity against a number of high priority bacterial pathogens. Screening led to the identification of four extracts with superior, broad-spectrum, antimicrobial activity. The extracts chosen were from *M. africana, P. papyracea, M. unguiculata,* and *D. herbacea*; a plant organism and three marine sponges. Bioassay-guided fractionation of the extracts led to the isolation of the active component in each extract. Extracts from *M. africana, P. papyracea, and D. herbacea* each led to the isolation and identification of a single, previously known compound; compounds 1, 2, and 4, respectively. The fractionation of the marine sponge *M. unguiculata* led to the isolation of a mixture of compounds, containing one previously unknown compound, monanchocidin F. All of the isolated active compounds displayed antimicrobial activity considered potentially useful for pharmaceutical development, ie. MIC's in the range of 0.02-10 μg/ml [98]. While all the compounds displayed antimicrobial activity on a variety of gram-positive and gram-negative bacteria, only the mixture of compounds in compound 3 was able to inhibit the growth of three species of gram-negative enterobaceriaceae *E. coli* 0157h7, *K. pneumoniae,* and *S. typhimurium*. After examining the ability of the isolated compounds to retain their antimicrobial effect

following thermal stress, and storage in various environments, it was found that compounds 3 and 4 were very stable, compound 1 was intermediate, and compound 2 was highly unstable. Finally, MTS assay proved that all the compounds were cytotoxic to the mouse embryonic cell line NIH 3T3.

In some cases, the extracts and isolated active compounds displayed similar levels of inhibitory activity against bacterial species. The loss of bioactivity is a common setback involved in isolation of bioactive components of extracts. Compounds 2 and 3 displayed inhibitory activity similar to the original extract of *P. papyracea* and *M. unguiculata*, respectively. Stability testing led to the conclusion that compound 2 is highly instable in nature. It is most likely that the degradation of compound 2 led to its decreased antimicrobial activity when compared with the original extract. For this reason, it would not be advisable to continue development of compound 2 as a natural antimicrobial. In the case of the mixture of compounds in compound 3, this mixture of compounds performed very well in stability studies and withstood environmental factors of degradation. This leads to the conclusions that the M. unguiculata extract may contain other antimicrobial components that were passed over during fractionation. It is likely that the mixture of compounds in compound 3, although highly active on their own, may work synergistically with other compounds in the extract. As compound 3 includes a previously undiscovered compound, which has been shown to be possess strong antimicrobial activity and stability, future directions for this work should focus heavily on the *M. unguiculata* extract. A small sample of extract was obtained for the purpose of this work. For future work, a larger sample of *M. unguiculata* may be obtained. This sample may be extracted and fractionated in a similar fashion to identify and isolate each active compound within the extract. Finally, the compounds may be combined in varying ratios to increase their antimicrobial activity. Although

compound 3 exhibited inhibitory activity on all of the bacterial species included in this study, further development should focus on its activity against the gram-negative enterobaceriaceae, *E. coli* 0157: H7, *K. pneumoniae,* and *S. typhimurium*. Further research should focus on elucidating the mechanism responsible for the antimicrobial activity of these compounds, as well as the mode of action responsible for their cytotoxicity. Molecular modification may be employed to further develop the lead compound.

Future work should also focus on the development of compound 1 as a replacement for the pharmaceutical agents used to control the bacterial species *A. baumannii*, and *L. monocytogenes.* Compound 1 displayed high antimicrobial activity against these two high priority pathogens which has not been described previously.

Compound 4 deserves further development as a natural antimicrobial as well. It could be developed as a replacement for currently used antibiotics for *B. subtilis*, *C. Jejuni*, *P. aeruginosa*, *S. aureus*, and *S. pneumoniae*. Compound 4 has potential as an antibmicrobial as it was highly active against a broad spectrum of bacterial species, it was a lead compound, displaying lower MIC's than the original extract, and it was found to be highly stable to stress and environmental factors. Future research should focus on the mechanism of action of the oxy-polyhalogenated diphenyl ether isolated from *Dysidea herbacea* as well as the mechanisms responsible for it's cytotoxicity against the MEF cell line. Chemical modification of this compound could lead to pharmaceutical development.

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF NATURAL ANTIMICROBIALS FROM PLANT AND MARINE ORGANISMS

by

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Advisor: Dr. Kevin Zhou

Major: Nutrition and Food Science

Degree: Doctor of Philosophy

Antibiotic agents have been found to have a limited lifespan due to the ability of microrganisms to develop antimicrobial resistance. In this study a number extracts of plant and marine organisms were evaluated for their antimicrobial activity against a number of high priority pathogens. Bioassay-guided fractionation of four extracts led to the identification of compounds with inhibitory activity; including one previously unknown compound. Compounds were evaluated through a variety of techniques including broth microdilution assay to determine each compound's MIC and MBC. The majority of the compounds showed antimicrobial activity equal to, if not better than, the commonly used antimicrobial drugs. The ability of compounds to withstand environmental stress and storage were explored with only one compound showing significant instability. Finally MTS assay showed that cell proliferation was decreased when mouse embryonic fibroblast cells, NIH 3T3 were treated with the compounds, proving they are cytotoxic to varying degrees.

AUTOBIOGRAPHICAL STATEMENT

I graduated from McGill University in 2007 with a Bachelor of Arts degree. In 2009 I completed my Honours Bachelor of Science degree in Foods and Nutrition at Brescia University College at The University of Western Ontario. I began my graduate studies at Wayne State University in 2009 as a Master's student in the Department of Nutrition and Food Sciences. In the summer of 2010 I was invited to transfer into the Ph.D. program working under the mentorship of Dr. K. Zhou. I am currently in my first year of a Post-Doctoral Fellowship at Ryerson University in the Department of Chemistry and Biology.